Reduced Immunogenicity of Arabidopsis hgl1 Mutant N-Glycans Caused by Altered Accessibility of Xylose and core Fucose Epitopes

Heidi Kaulfürst-Soboll, Stephan Rips, Hisashi Koiwa, Hiroyuki Kajiura, Kazuhiro Fujiyama, and Antje von Schaewen

From the Institut für Biologie und Biotechnologie der Pflanzen, Westfälische Wilhelms-Universität Münster, 48149 Münster, Germany, the Plant Science Program, Texas A & M University, College Station, Texas 77843-2133, and the International Center for Biotechnology, Osaka University, 2-1 Yamada-oka, Suita-shi, Osaka 565-0871, Japan

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 β1,2-xylose and α1,3-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-β1,2-xylose and anti-α1,3-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 α1,6-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-acetylglucosaminylytransferase 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), play pivotal roles in stress tolerance and plant development (stauroporin 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, β1,2-xylosyltransferase (XylT), α1,3-fucosyltransferase (FucTa, FucTb), and N-acetylglucosaminyltransferase II (GnTII), which altogether produce N-glycan structures containing β1,2-xylose and/or core α1,3-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 β1,3-galactosyltransferase and α1,4-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 mannioside II mutant, equivalent to hgl1) retain branched mannoses on their α1,6-arm and produce N-glycans containing xylose as well as core fucose residues (Fig. 1).

Plant-derived complex N-glycans with β1,2-xylose and/or α1,3-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 carbohydrate determinants; Ig, immunoglobulin; Mil, α-mannosidase II; PHA-L, phytohemagglutinin-L; GN, N-acetylglucosaminidase; MES, 2-(4-Morpholino)ethanesulphonic acid; CTG, N-acetylglucosaminylytransferase I; GnTI, and often responsible for IgE recognition of several glycoallergens.
carbohydrate determinants (CCD) (14). In addition to hymenoptera venom-allergic patients, CCD-specific IgE antibodies are notoriously present in the sera of pollen-sensitized individuals and patients with plant food allergies. By contrast to IgG, occurrence of IgE antibodies indicates allergic sensitization. However, it is still a matter of debate whether invertebrate- and plant-derived CCD epitopes are causal agents or clinically irrelevant (reviewed in Refs. 15 and 16). Recent evidence suggests that CCD epitopes may function as Th2 adjuvant during manifestation of allergic reactions (Ref. 17 and references cited therein).

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).

**FIGURE 1.** Schematic representation of N-glycosylation steps in the ER and further N-glycan modification in the Golgi apparatus of plant cells. Steps that produce N-glycans recognized by complex glycan-specific antibodies are shaded gray. Consequences of steps blocked in Arabidopsis cgl1 (GnTI) and hgl1 (manII; ManII) mutants are also depicted in gray. The dotted lines indicate terminal residues removed by mannosidase(s) (49) or GlcNac-specific hexosaminidases in vacuoles or the cell wall (45). The latter seems to occur less efficiently in the apoplast of leaf tissue analyzed here (46). The fully processed, complex N-glycan of wild type is circled by a dashed line. Note that in agreement with three-dimensional N-glycan models (15) (compare Fig. 3B), we depict core α1,3-fucose lying opposite of β1,2-xylose. The glycan symbols are according to ProGlycAn. For details on N-glycan structures see supplemental Fig. S3.
Arabidopsis hgl1 Mutants Are Hypoallergenic

well studied immunologically using crude antiserum and antibody fractions purified thereof that bind independently to β1,2-xylose and α,1,3-fucose epitopes (22). Such complex-glycan antiserum 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-fructofuranosidase (26, 27); pata- tin, 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 steric hindrance caused by the presence of α,1,3-mannose in case of M3XF (MMXF), whereas Bencu´rova´ 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 antiserum 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(e) antibodies (Kirkegaard & Perry Laboratories, Gaithersburg, MD) diluted 1:10,000 in TBST for 1 h, followed again by wash steps. Concanavalin A affinoblotting 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 antiserum. Total soluble protein was extracted from leaves of Arabidopsis cgl1, xylT (B1,2-xylotransferase), 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-FucHRP or α-XylHRP) 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-XylPHA-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 μg, 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 μg 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 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 cgll) 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
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 (H9251-XylPHA-L and H9251-XylHRP) substantiated this assumption: H9251-XylHRP 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\textsubscript{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 H9251-XylHRP, respectively. Based on this nomenclature, A form antibodies should have similar epitope specificity as H9251-XylPHA-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

| N-Glycan structure | Ratio |
|--------------------|-------|
| WT (Col-0) fucTab xylT hgl1-1 (exon) hgl1-2 (intron) hgl1 fucTab hgl1 xylT |
| % |
| Mannose-type |
| M3 | 6.9 | 7.0 |
| M4 | 6.9 | 7.0 |
| 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 | 6.0 | 3.2 | 5.0 | 1.5 | 3.5 | 1.2 | 3.3 |
| GlcNac-terminated |
| GmM3 | 2.5 | 2.6 |
| GmM5 | 2.0 | 3.2 | 4.9 | 19.2 |
| GnM2M3 | 2.1 |
| Complex-type (fucose-linked) |
| M3F | 19.0 |
| M4F | 3.5 | 1.9 |
| M5F | 1.9 |
| Complex-type (xylose-linked) |
| M3X | 5.7 | 30.5 |
| M4X | 2.6 |
| M5X | 4.9 | 7.5 | 35.4 |
| Complex-type (fucose- and xylose-linked) |
| M3XF | 24.9 |
| M4XF | 2.7 |
| M5XF | 24.0 | 22.3 |
| Complex-type (GlcNac-terminated, fucose and/or xylose-linked) |
| GmM3F | 7.2 |
| GmM4F | 7.2 |
| GmM5F | 13.1 |
| GmM3X | 6.6 |
| GmM3X | 2.1 | 6.1 |
| GmM2M3X | 7.3 |
| GmM4X | 2.9 |
| GmM5X | 5.1 | 7.3 | 32.5 |
| GmM3XF | 7.2 |
| GmM4XF | 7.9 |
| GmM5XF | 21.1 |
| 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 |

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

Arabidopsis hgl1 Mutants Are Hypoallergenic
Arabidopsis hgl1 Mutants Are Hypoallergenic

FIGURE 3. Xylose-specific binding of two complex glycan antisera differs as revealed by comparison of hgl1 xylT and hgl1 fucTab extracts. A, protein extracts (20 μg/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-XylPHA-L or α-XylHRP). Prominent differences between α-XylPHA-L and α-XylHRP in binding xylene 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 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 conformation was also predicted for singly GlcNac-terminated glycans (GnM3XF; Table 1). These three-dimensional models suggested that β1,2-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 β1,2-xylose epitopes can vary, depending on the presence or absence of terminal GlcNac and/or mannose residues on α13- and α16-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 α1,3-mannosidase treatment. To favor glycan-specific (and to avoid peptide-based reactions), affinity-selected antibodies of PHA-L and HRP antisera (α-XylPHA-L and α-XylHRP, respectively) were used as probes. A fucose-specific HRP fraction (α-FucHRP) 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 α13-mannose (Fig. 3B). Thus, α13-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, α13-mannosidase treatment of PHA-L decreased xylose-specific binding (Fig. 4, bottom panel). Noteworthy, fucose-specific antibody binding (α-FucHRP) was not affected by the treatment, and concanavalin A affinoblotting 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 α13-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-XylPHA-L and α-XylHRP antibodies (Fig. 5), indicative of converting Gn(2)M3XF (F) to fully processed M3XF (F) 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 α16-arm. Importantly, Golgi α-mannosidase II (MII) can
only release those mannoses when the α1,3-antenna is decorated with 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, xylose 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 α1,6-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 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

---

**FIGURE 4.** Modulation of xylose conformation by α1,3-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 α1,3-mannosidase (exoenzyme; New England Biolabs) prior to 12% SDS-PAGE (1 μg of PHA-L loaded per lane) and immunoblot development of separate blot strips. Xylose-specific and fucose-specific antibody fractions (α-XylPHA-L, α-XylHRP, and α-FucHRP) 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.

**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 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 μg 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.
Arabidopsis hgl1 Mutants Are Hypoallergenic

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 plants 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 hexosaminidases.
dases residing in vacuoles or in the cell wall (45, 46). Alternatively, N-glycans with untrimmed α1,6-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 α1,3-mannose on M3XF(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 α1,3- and/or α1,6-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 α1,3- and α1,6-linked mannos (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 α1,6-linked manno(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-β1,2-xylose antibodies. Apart from the sterical specificity of the antibodies proposed here, binding of α1,3-mannose linked to the manno 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 Bencu´rova´ 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-XylPHL,s 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 α1,6-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 mannos 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 antisem when terminal mannos are lost. Similarly, Kurosaka et al. (30) proposed that binding of fucose-specific HRP antibodies involves α1,6-linked manno. Consequently, additional sugar linkages on the α1,6-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 α1,6-arm are not highly abundant in wild type (compare Table 1), and treatment of wild type extracts with N-acetylgalcosaminidase 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 α1,6-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 α1,6-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 β1,2-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-acetylgalactosaminyltransferase 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-acetylhexosamidase activities recently suggested by Liebming 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.

Acknowledgments—Initial support by Julia Frank and the skilled technical assistance of Kerstin Fischer (Münster, Germany) are gratefully acknowledged. We thank Doug Kuntz (Ontario Cancer Institute at MaRS, Toronto, Canada) for generous gifts of purified recombinant Drosophila Golgi α-mannosidase II enzyme, and Melanie Mertens (University Hospital Münster Department of Dermatology, Allergology) for CCD-specific patient sera and comments on the manuscript. The Arabidopsis Information Resource (TAIR) was invaluable for our work with Arabidopsis mutants.

REFERENCES

1. Koiva, H., Li, F., McCully, M. G., Mendoza, I., Koizumi, N., Manabe, Y., Nakagawa, Y., Zhu, J., Rus, A., Pardo, J. M., Bressan, R. A., and Hasegawa, P. M. (2003) Plant Cell 15, 2273–2284
Arabidopsis hgl1 Mutants Are Hypoallergenic

24. Kaladas, P. M., Goldberg, R., and Poretz, R. D. (1983) Mol. Immunol. 20, 727–735
25. Ramirez-Soto, D., and Poretz, R. D. (1991) Carbohydr. Res. 213, 27–36
26. Faye, L., and Chrispeels, M. J. (1988) Glycoconj. J. 5, 245–256
27. Sturm, A., and Chrispeels, M. J. (1990) Plant Cell 2, 1107–1119
28. Sonnewald, U., Studer, D., Rocha-Sosa, M., and Willmitzer, L. (1989) Planta 178, 176–183
29. Laurière, M., Laurière, C., Chrispeels, M. J., Johnson, K. D., and Sturm, A. (1989) Plant Physiol. 90, 1182–1188
30. Kurosaka, A., Yano, A., Itoh, N., Kuroda, Y., Nakagawa, T., and Kawasaki, T. (1991) J. Biol. Chem. 266, 4168–4172
31. Bencúrová, M., Hemmer, W., Focke-Tejkl, M., Wilson, I. B., and Altmann, F. (2004) Glycobiology 14, 457–466
32. Kajíra, H., Seki, T., and Fujiyama, K. (2010) Glycobiology 20, 736–751
33. Tomiya, N., Lee, Y. C., Yoshida, T., Wada, Y., Awaya, J., Kurono, M., and Takahashi, N. (1991) Anal. Biochem. 193, 90–100
34. Wenderoth, I., and von Schaewen, A. (2000) Plant Physiol. 123, 1097–1108
35. Faye, L., and Chrispeels, M. J. (1985) Anal. Biochem. 149, 218–224
36. Kaulfurst-Soboll, H., Mertens, M., Brehler, R., and von Schaewen, A. (2011) PLoS ONE 6, e17800
37. Shah, N., Kuntz, D. A., and Rose, D. R. (2008) Proc. Natl. Acad. Sci. U.S.A. 105, 9570–9575
38. Sturm, A., and Chrispeels, M. J. (1986) Plant Physiol. 81, 320–322
39. Brewer, C. F., and Bhattacharyya, L. (1986) J. Biol. Chem. 261, 7306–7310
40. van den Elsen, J. M., Kuntz, D. A., and Rose, D. R. (2001) EMBO J. 20, 3008–3017
41. Dennis, J. W., Nabi, I. R., and Demetriou, M. (2009) Cell 139, 1229–1241
42. De Hoft, P. L., Beill, L. M., and Hirsch, A. M. (2009) Mol. Genet. Genomics 282, 1–15
43. Bencúr, P., Steinkellner, H., Svoboda, B., Mucha, J., Strasser, R., Kolarich, D., Hann, S., Köllensperger, G., Glössl, J., Altmann, F., and Mach, L. (2005) Biochem. J. 388, 515–525
44. Saint-Jore-Dupas, C., Nebenführ, A., Boulaflous, A., Follet-Gueye, M. L., Plasson, C., Hawes, C., Driouich, A., Faye, L., and Gomord, V. (2006) Plant Cell 18, 3182–3200
45. Strasser, R., Bondili, J. S., Schobrer, J., Svoboda, B., Liebinger, E., Glössl, J., Altmann, F., Steinkellner, H., and Mach, L. (2007) Plant Physiol. 145, 5–16
46. Liebinger, E., Veit, C., Pabst, M., Batoux, M., Zipfel, C., Altmann, F., Mach, L., and Strasser, R. (2011) J. Biol. Chem. 286, 10793–10802
47. Lommerse, J. P., Kroon-Batenburg, L. M., Kroon, J., Kamerling, J. P., and Vliegenthart, J. F. (1995) J. Biol. Chem. 270, 11451–11458
48. Wuhrer, M., Balog, C. I., Koeliman, C. A., Deelder, A. M., and Hokke, C. H. (2005) Biochim. Biophys. Acta 1723, 229–239
49. Faye, L., Gomord, V., Fitchette-Lainé, A. C., and Chrispeels, M. J. (1993) Anal. Biochem. 209, 104–108
50. Prenner, C., Mach, L., Glössl, J., and März, L. (1992) Biochem. J. 284, 377–380