

Comparative mass spectrometry analysis of N-glycans from the glycoproteins of eight allergy-inducing plants

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ABSTRACT

Extensive cross-allergic reactivity exists among food-borne plants. As most allergens are glycoproteins, protein sensitization has been extensively studied. However, little attention has been given to the glycan component of glycoproteins. Here, we qualitatively and quantitatively compared the N-glycans of eight cross-reactive plant glycoproteins derived from peach pollen, soy, pine nut, cashew nut, pistachio, walnut, almond, and hazelnut. Cross-reactive carbohydrate determinants (CCDs) were widely present in the allergic glycoproteins according to the following order: pollen > soybean > pistachios > pine nuts > walnuts > cashews > hazelnuts > almonds. The N-glycan structure presented clear differences between the germplasm and vegetative tissue. Importantly, fucosylation and xylosylation levels correlated positively with extensive cross-reactivity between plants, which suggests that CCDs may be one of the causes of cross-allergy. This result provides the foundation for mechanistic studies on plant-derived CCDs as a source of food cross-allergies.

1. Introduction

Food allergy represents an abnormal immune response triggered by the ingestion of certain foods, which may be accompanied by other allergic diseases, such as asthma, urticaria, atopic dermatitis, allergic rhinitis, and eosinophilic esophagitis (Flora-Glad et al., 2018; Leung et al., 2014). The prevalence of food allergies is steadily increasing, having become a serious economic burden and a substantial public health concern worldwide (Moneret-Vautrin et al., 2005).

Food allergy is generally mediated by immunoglobulin E (IgE) and usually occurs within 30–60 min after ingestion. The allergens in food become exposed through digestion, enter the gut lumen and, once they encounter the gut barrier, they are captured by dendritic cells, processed, and released to Th2 cells in a process mediated by the major histocompatibility complex. Subsequently, Th2 cells promote B cells to release specific IgEs to bind to the Fc receptor on mast cells or basophils, thereby activating these two types of cells. When activated mast cells or basophils encounter again the food allergen, IgEs present on the surface of basophils and mast cells bind to the antigen and cross-link the

receptors, releasing a series of histamine-derived inflammatory factors that trigger the allergic reaction (Cianferoni and Spergel, 2009). Co-ingestion of certain foods may cause more severe allergies due to cross-reactivity (Matthew et al., 2010). This is due to the ongoing presence of mast cells or basophils displaying IgEs after the first allergic response. When encountering a known antigen, IgEs will immediately bind to the antigen and cause the receptor to cross-link to release histamine, thus triggering a renewed allergic response.

Common food allergens include milk, eggs, soybeans, peanuts, tree nuts, buckwheat, fish, and shellfish (Shriver and Yang, 2011). Among them, tree nuts can cause serious food allergies, which can threaten the health of young children and have an adverse effect on adults (Bock et al., 2001; Branum and Lukacs, 2009). According to statistics, cross-sensitivity between various nuts can reach 92%, and a value of 59–86% has been reported between peanut and soybean (Matthew et al., 2010). Cross-allergic reactions can occur also between plant-derived foods and pollen, as suggested by 70% of patients allergic to pollen experiencing allergy symptoms when consuming nuts and soybeans (Vieths et al., 2002). Studies have taken advantage of cross-reactivity

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between plant-derived food allergens to try desensitization treatments and have found that patients allergic to cashews and pistachios developed immune tolerance to pistachios after cashew immunotolerance treatment (Elizur et al., 2019). Therefore, the systematic study of allergens present in food-derived plants will help us understand the causes of cross-reactivity and improve immune desensitization treatment.

Plant-derived foods capable of causing cross-allergies are collectively called pan-allergens. The reactions caused by pan-allergens are usually limited to the oropharynx and oral cavity, but in a few cases can cause systemic allergic reactions (Kleine-Tebbe et al., 2002; Asarnej et al., 2012). Studies on the conformation of pan-allergens have pointed to homologous proteins, such as the Jug r 5 and Cor a 1 families in nuts and the Bet v 1 family in pollen (Price et al., 2015). Homologous lipid transfer protein families have been identified in walnuts (Jug r 3), hazelnuts (Cor a 8), and almonds (Pru du 3) (Wangorsch et al., 2017; Hofmann et al., 2013). Highly homologous and cross-reactive Ana o 1 and Pis v 3 proteins have been identified in cashews and pistachio nuts, respectively (Costa et al., 2019).

In addition to homologous proteins, antigen-sharing IgE-binding epitope regions may also cause cross-reactivity. *N*-glycans with core α -1,3-fucose and β -1,2-xylose modifications are present on plant glycoproteins and contain binding sites specific to IgE. These are known as cross-reactive carbohydrate determinants (CCDs) and cause the body to produce specific IgEs associated with extensive cross-reactions between plants. Studies have found that silencing β -1,2-xylosyltransferase-related genes in plants can reduce allergic reactivity (Aalberse et al., 1981; Foetisch et al., 2003; Jappe et al., 2006; Scott, 2015). Monovalent CCDs will not cause severe allergic reactions, but multivalent CCDs will increase the degree of binding to s-IgEs and trigger extensive histamine release, which directly affects the degree of sensitization (Bencurova et al., 2004). Considering the scarcity of comparative studies describing changes in structure and content of CCDs in different plants, detailed analysis of *N*-glycans from plant food glycoproteins could provide novel information on their role in allergic reactions.

Here, glycoprotein *N*-glycans of soybean, pollen, and nuts from eight different plant taxa were qualitatively and quantitatively compared by electrospray ionization mass spectrometry (ESI-MS) and online liquid phase-multi-stage tandem mass spectrometry (LC-MS/MS). The information thus obtained will advance our understanding of the relationship between CCDs and plant cross-reactivity.

2. Materials and methods

2.1. Materials and reagents

1-phenyl-3-methyl-5-pyrazolone (PMP) was purchased from Aladdin. Ammonium sulfate, methanol, acetonitrile, ammonia, and dichloromethane were all of analytical grade and were purchased from Ruilijie; Instrumentation included a DD-5 M cryogenic centrifuge (Hunan Xiangyi Centrifuge Instrument Co., Ltd.), ultra-low temperature HetoPowerDry LL3000 vacuum freeze dryer (Denmark Heto-Holten), porous graphitic carbon (PGC) column (150 mg/4 mL; Alltech Associates), C18 column (250 mg/mL; Waters), cotton column (100 mg; Northwest University Hospital), and LTQ-XL electrospray ionization mass spectrometer (Thermo Scientific).

2.2. Sample collection

Allergy-inducing samples derived from both gymnosperms (pine nuts) and angiosperm dicotyledonous plants (soybeans, hazelnuts, cashews, pistachios, walnuts, almonds, and pollen) were purchased from Lijiacun Farmers Market, Xi'an City, Shaanxi Province, PRC.

2.3. Extraction of total protein extraction.

Pine nuts, walnuts, pistachios, cashews, hazelnuts, soybeans, and

almonds were dried in an oven, and then crushed to a powder form with a multifunctional grinder. The powder was mixed with petroleum ether at a ratio of 1:4 (m/v) and degreased at 4 °C for 3 h. The upper layer of petroleum ether was removed by centrifugation at 4 °C (6000×g, 10 min), and the operation was repeated three times until the liquid became colorless. Petroleum ether was removed and the resulting defatted powder was mixed at a ratio of 1:15 (w/v) with a protein extraction solution consisting of 0.03 M Tris-HCl (pH 7.6). The mixture was stirred at 4 °C for 2 h, the supernatant containing the proteins was collected by centrifugation at 4 °C (6000×g, 10 min), the pellet was mixed again with the protein extraction solution, and the extraction procedure was repeated. Finally, the two protein solutions were mixed together (Nagano et al., 1996; Venkatachalam and Sathe, 2006; Mao and Hua, 2012; Al-Ismael et al., 2018). Lyophilized pollen (10 g) was added to sufficient liquid nitrogen and pulverized with 50 mL 0.1 M sodium phosphate buffer (pH 7.5), the supernatant containing the proteins was collected by centrifugation at 4 °C (1500×g, 2 min), and the pellet was extracted repeatedly as detailed above. The two protein solutions were mixed together (Giavalisco et al., 2003). Finally, each protein sample was precipitated by ammonium sulfate salting; after which total protein was dissolved in buffer solution, dialyzed for 3 days, and lyophilized for subsequent use.

2.4. Release and purification of *N*-glycans

Release of *N*-glycans was started by dissolving 10 mg glycoprotein sample in 4 mL of 25% aqueous ammonia (v/v) in a sealed vessel before letting the reaction proceed for 16 h at 60 °C in a water bath. After the reaction was completed, the contents were cooled to room temperature and the ammonia water was evaporated with a rotary evaporator. Then, the dried sample was redissolved in 2 mL deionized water and the pH was adjusted to about 7 using 25% aqueous acetic acid solution (v/v). The solution was dried in a SpeedVac concentrator prior to purification with C18/PGC solid-phase extraction (SPE) and cotton columns (Wang et al., 2018). Purification via the C18 SPE column was as follows: the column was prewashed with 3 mL acetonitrile and 10 mL deionized water, the sample was loaded repeatedly three times onto the column, and finally glycans were eluted with 20 mL deionized water.

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The eluates from the C18 SPE column were collected for PGC SPE column purification. First, the eluates were loaded onto the column prewashed as above; then, salt was washed with 10 mL deionized water; and, finally, the sample was eluted with 3 mL of 25% acetonitrile aqueous solution (v/v) and collected for MS detection (Yuan et al., 2014).

The eluate containing the target glycan was combined and dried, redissolved in 100 μ L deionized water, and loaded on the cotton column. After the solution was completely absorbed, the column was dried in a vacuum oven. Excess impurities were washed with 10–20 mL acetonitrile, followed by a gradient of 90%, 85%, and 80% acetonitrile aqueous solution (v/v), and 400 μ L of 50% acetonitrile aqueous solution (v/v). Finally, deionized water was used to elute the reducing glycan: 200 μ L per tube for a total of 10 tubes were collected for ESI-MS analysis and dried for derivatization (Peng et al., 2019).

2.5. Derivatization of glycans with PMP

Briefly, 1 mL of 0.5 M PMP-methanol solution and 1 mL of 0.3 M NaOH aqueous solution were added to pure reducing *N*-glycans in a hermetic glass tube. After fully dissolving and mixing the contents, these were reacted for 90 min at 70 °C in a water bath. Once the reaction was completed, the samples were neutralized by adding acetic acid before three rounds of extraction with 1 mL dichloromethane to remove excess

PMP and then concentrated to complete dryness (Wang et al., 2018). The samples were redissolved in 1 mL deionized water prior to loading onto the C18 PGC column prewashed with 3 mL acetonitrile and 10 mL deionized water for purification; then, salt was washed with 15–20 mL deionized water; and, finally, the target glycan derivatives were eluted with 3 mL of 25% acetonitrile aqueous solution (v/v) and concentrated for subsequent LC-MS analysis (Yuan et al., 2014).

2.6. Relative quantification of N-glycans

First, 2 mg Gal-Ara standard disaccharide was derivatized with PMP as described in Section 2.4. Next, the Gal-Ara-2PMP derivative was diluted 1000 times and divided into 24 equal parts, which were added to N-glycan derivatives so that each sample contained the same amount of

standard. After LC-MS analysis, the relative content of all target N-glycans in the different samples was obtained by calculating the ratio of the peak area of the extracted target N-glycan to the peak area of the standard. This value facilitated the relative quantitative comparison of N-glycans between samples and within the samples themselves.

2.7. ESI-MS analysis

ESI-MS analysis of glycans was performed on a Thermo LTQ-XL tandem four stage linear ion trap mass spectrometer coupled with an electrospray ion source. The procedure was carried out in positive ion mode as follows: spray voltage of 4 kV, with a sheath gas (N₂) flow rate of 20.0 arb, an auxiliary gas (N₂) flow rate of 10.0 arb, a capillary temperature of 300 °C, a capillary voltage of 37 V, a capillary lens

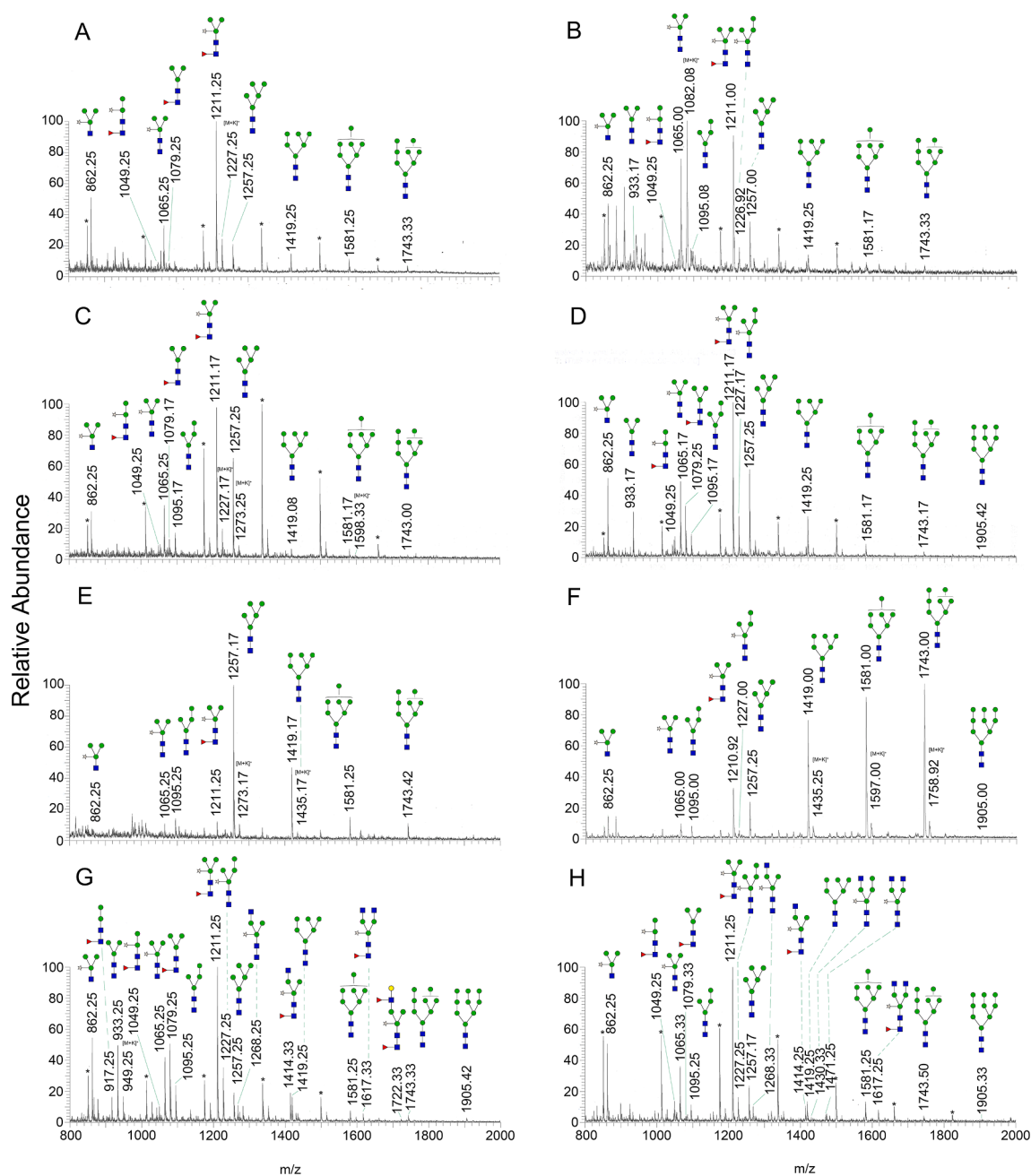


Fig. 1. ESI-MS analysis of N-glycans from eight plant glycoproteins. (A) Cashew; (B) Hazelnut; (C) Pistachio; (D) walnut; (E) almond; (F) soybean; (G) peach pollen; (H) pine nut. * indicates glucomannan. Structural formulae: blue square, N-acetylglucosamine; orange stars, xylose; green circle, mannose; red triangle, fucose. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

voltage of 250 V, sample injection of 2 μ L, mobile phase consisting of 50% methanol, and sample flow rate of 20 μ L/min.

2.8. LC-MS/MS detection

The Penta-HILIC column (2.1 mm $\times$ 150 mm, 5 μ m) was used with a column temperature of 40 $^{\circ}$ C, UV detection at 254 nm, flow rate of 0.3 mL/min, and injection volume of 2 μ L. Mobile phase A was acetonitrile, B was 100 mM ammonium acetate solution (pH 6.0), and C was double-distilled water. Sample separation conditions were as follows: $t = 0$ min, 90% A, 10% B, 1 mL/min; $t = 120$ min, 90% A, 10% B, 1 mL/min. The chromatographic peaks eluted from the column were separately analyzed in the ESI-MS/MS system by changing rapidly between positive form MS and MS/MS fragments based on data dependency capture with collision energy of 30 V and minimum signal strength of 500. For MS/MS analysis, *N*-glycans were subjected to fragmentation by collision-induced dissociation, with helium (He) as the collision gas, isotope width of m/z 3.0, and normalized collision energy of 30–60. Other parameters for MS and MS/MS were as those for ESI-MS. Data were acquired by Xcalibur software (Thermo Scientific) and analyzed using GlycoWorkbench software (Ceroni et al., 2008).

3. Results and discussion

3.1. ESI-MS analysis of reducing *N*-glycans from eight plant glycoproteins

The ammonia method established by our laboratory was used to release *N*-glycans from plant-derived glycoproteins (Wang et al., 2018). The resulting reducing *N*-glycans were purified by C18 and PGC SPE columns, as well as a cotton column. (Peng et al., 2019) Fig. 1 shows the ESI-MS spectrum of reducing *N*-glycans from eight plant glycoproteins. The abundance of *N*-glycans varied between plant species, with almond (Fig. 1E) and soybean (Fig. 1F) presenting the lowest number of reducing *N*-glycans, and pollen (Fig. 1G) and pine nut (Fig. 1H) presenting the highest.

N-glycans carrying core α -1,3-fucose and β -1,2-xylose were previously reported to associate with anti-CCD IgE and cause allergic reactions (Ronald et al., 2000). Fig. 1 that plant glycoproteins generally contained CCDs with two structures, MMXF (m/z 1211) and MMX (m/z 1065). In addition, we found differences in the fucosylated and xylosylated glycoform structures of different plant-derived glycoproteins. Specifically, as a gymnosperm plant, pine nuts contained two unique xylosylation structures (Man4GnX (m/z 1430.33) and GnGnX (m/z 1471.25)), which were absent from angiosperm nuts (Fig. 1H). Second, whereas nuts originate from seeds, pollen belongs to floral organs and here appeared rich in fucosylated structures (MUF (m/z 917.33) and M (GF)XF (m/z 1722.33)), which were not detected in seeds (Fig. 1D). These results contrast somewhat with evidence of α -1,3-fucose-modified and β -1,2-xylose-modified *N*-glycans in plant-derived glycoproteins (Richard et al., 2016). Overall, Fig. 1 indicate variation in the structure of glycoprotein CCDs from different plant sources.

3.2. Online LC-MS/MS analysis of eight plant glycoprotein *N*-glycan derivatives

To distinguish the isomers of eight plant-derived glycoprotein *N*-glycans and improve their MS detection sensitivity, the obtained reduced *N*-glycans were subjected to PMP derivatization and used for LC-MS/MS analysis.

LC-MS/MS spectra of hazelnuts, walnuts, soybeans, almonds, cashews, pistachios, pine nuts, and pollen glycoprotein *N*-glycan derivatives are presented in Figures S1–S8. As shown in Figures S1 and S4, derivatization of reduced *N*-glycans from hazelnut and almond glycoproteins led to a greater abundance of the MMF (pentose core modified by α 1,3-fucose) *N*-glycan structure. As shown in Figure S8, derivatization of the reduced *N*-glycans from pollen glycoproteins allowed the

detection of an additional *N*-glycan structure, MGnF, which consisted of a pentose core modified by α 1,3-fucose plus a non-reducing end modified by *N*-acetylglucose.

Table 2 shows that the CCDs of cashews and pistachios shared the same structure, which consisted of one truncated MUXF moiety and three MMX/F/XF complexes. The CCDs of walnuts, pine nuts, and hazelnuts shared the same structure as cashews and pistachios with the addition of a hybrid Man4X. Five types of non-reduced *N*-acetylglucose-modified CCDs were detected in the *N*-glycan derivatives of pine nuts than other nuts. Three types of CCDs were detected in almonds, and all were consistent with complexes seen also in hazelnuts, walnuts, cashews, and pistachios. Soybeans presented three types of CCDs, two MMX/XF complexes and one hybrid type consistent with that of hazelnuts, pine nuts, and walnuts. Pollen displayed seven kinds of CCDs: truncated CCDs had an extra MUF structure compared to nuts, complexed CCDs had one more Lewis structure (M(GF)XF), and hybrid CCDs were consistent with those found in hazelnuts, walnuts, and pine nuts. The non-reduced *N*-acetylglucose-modified xylosylated complex (GnGnX) was not detected in pollen glycoprotein *N*-glycans; however, a non-reduced *N*-acetylglucose-modified fucosylated structure (MGnF) was more abundant in pollen than in pine nuts.

Based on the extracted ion chromatogram (EIC) displayed in Figure S7B, pine nuts glycoprotein *N*-glycan derivatives can be seen to display two pairs of peaks with the same molecular weight but different structure (m/z 1395.33 and 1598.33). Three pairs of peaks with the same molecular weight but different structures (m/z 1263.33, 1395.33, and 1598.33) corresponding to *N*-glycan derivatives from pollen glycoproteins can be observed in the EIC in Figure S8B. To further identify these structures, MS/MS was combined with GlycoWorkbench analysis (Ceroni et al., 2008). As shown in Figure S9, none of the glycan structures had a complete pentose core, which was hypothesized to correspond to the glycan structure at m/z 1192.33 (reducing *N*-glycan $m/z = 862$), indicating Peeling degradation by-products introduced during ammonia hydrolysis. Indeed, m/z 1192 is the degradation by-product of MMXF, m/z 1263.33 is the degradation by-product of MGnF, m/z 1395.33 is the degradation by-product of MGnXF, and m/z 1598.33 is the degradation by-product of GnGnXF. We previously reported that when ammonia was used to release core α -1,3-fucosylated *N*-glycans, the β -1,4 glycosidic bond between the two *N*-acetyl glucoses close to the amino acid was prone to be partially broken, a phenomenon known as Peeling degradation; however, this degradation would only occur once and would not continue to deglycosylate (Wang et al., 2018). Using LC-MS, the core α -1,3-fucosylated *N*-glycan and its Peeling degradation product can be integrated at the same time and combined for accurate quantification of glycans.

3.3. Relative quantitative analysis of *N*-glycans from eight plant glycoproteins

The degree of cross-allergic reaction varies between different foods. For example, soybean is susceptible to cross-allergy with tree nuts. To compare the relationship between the various CCD contents in different plants and cross-allergic reactions, quantitative analysis of eight plant-derived glycoprotein *N*-glycans was performed. Absolute quantification of glycan samples is normally based on well-characterized glycan standards. However, trace glycans from various organisms can be very complex and, in most cases, commercial glycan standards are not available. Usually, oligosaccharide standards are selected as a reference for relative quantitative analysis of target glycan samples (Wang et al., 2011; Zhao et al., 2015). Adding the internal standard directly to the glycan sample is the simplest and most convenient method (Zhou et al., 2016; Walker et al., 2013). Therefore, to achieve the relative quantification of various types of glycans between samples, we added the internal standard glycan derivative (Gal-Ara-2PMP) directly to the glycoprotein *N*-glycan derivatives.

The distribution and content of different types of *N*-glycans from

eight plant-derived glycoproteins are shown in Fig. 2 and Table 1. MMXF and MMX content in eight plant-derived glycoproteins was as follows: pollen > soybean > pine nuts > walnuts > pistachios > cashews > hazelnuts > almonds. High-mannose types (Man5–8) were present in glycoproteins from all eight plant species, with the highest content being in soybean. A low content of MUF, MGnF, and M(GF)XF could be detected in peach pollen glycoproteins. These last three glycoforms represent fucosylated *N*-glycans. Modification of the non-reducing end with *N*-acetyl glucose has been shown to mask its own sensitization, so MGnF may not cause serious allergic reactions (Bencurova et al., 2004). M(GF)XF is a Lewis structure and is thought to cause severe allergic reactions (Commings et al., 2009). The xylosylated *N*-glycans Man4GnX and GnX were detected only in pine nut proteins; as they are both modified by *N*-acetyl glucose at the non-reducing end, they do not display severe sensitization. The non-reducing end of the *N*-glycan modified by *N*-acetyl glucose included also MGnX, MGnXF, and GnGnXF, all of which were found in pollen and pine nuts, but not in other nuts or soybean glycoproteins. As shown in Fig. 2, high-mannose *N*-glycans were dominant in soybean glycoproteins; whereas fucosylated and xylosylated *N*-glycans were predominant in pollen glycoproteins. Xylosylation was elevated also in pine nut glycoprotein *N*-glycans, whereby it was second only to pollen.

Based on their potential antigen recognition site, glycans were divided into three types: potentially sensitized (xylosylated, fucosylated, both fucosylated and xylosylated), closed sensitization type (*N*-acetyl glucose modification of the non-reducing end), and high-mannose type. Relative quantitative comparison of different *N*-glycan types (Fig. 3A), revealed that CCDs accounted for 80% of cashew glycoproteins and 81% of pistachio glycoproteins. Cashews and pistachios belong to the Anacardiaceae family, which may explain the minimal difference in composition. In contrast, peach pollens and almonds also belong to the same family of Rosaceae, yet CCD content was 49% higher in peach pollen than in almonds, and pollen was more prone to cross-allergy (Fig. 3B). This finding points to germplasm-specific vs. organ-specific CCDs in plants. This difference may arise from species and organ selectivity of glycosyltransferase during endoplasmic reticulum glycosylation, which may lead to glycoproteins carrying CCDs being more likely to cause extensive cross-allergies (Richard, 2016; Strasser et al., 2007). The closer the kinship is, the closer the CCDs structure and glycosylation level are. We also found that floral organs seemed to be more prone to synthesize α -1,3-fucose-modified CCDs than seeds. We speculate that these sensitized CCDs may represent a conserved trait in

plants and play an important biological function. A sudden change in glycosylation selectivity, may lead to loss of certain functions, dysplasia, nutritional loss, and ultimately loss of its edible value.

Here, comparative quantitative analysis of *N*-glycan derivatives from different sources has revealed that a broad cross-allergic reaction is related to the abundance of plant glycoprotein CCDs. This is illustrated clearly in Fig. 3A by the 99% similarity between cashew and pistachio glycoprotein CCDs, and is in line with the high cross-allergy triggered by these two types of nuts. Studies have found 79% similarity between allergenic protein sequences in cashews and pistachios (Costa et al., 2019). Cross-allergic reactions were reported also between cashew and walnut in a mouse model (Kulis et al., 2009), and were confirmed here by 89% similarity between CCDs from these two species (Fig. 3C). As no homologous protein was found between the two nuts, protein sequence similarity is clearly not the only reason for cross-allergic reactions. Clinical data have revealed high cross-allergy levels between almond and hazelnut, as well as the existence of homologous proteins (Branum and Lukacs, 2009; Hofmann et al., 2013); however, here, CCDs from these two species exhibited only 39% similarity (Fig. 3D). Arnon et al. found a certain degree of cross-reaction between hazelnut and walnut, and others confirmed the existence of homologous proteins (Elizur et al., 2019; Price et al., 2015; Wangorsch et al., 2017). Here, we show that CCDs from the two species shared 86% similarity (Fig. 3E). Based on this evidence, we speculate that the abundance of plant glycoprotein CCDs from different sources is closely related to the cross-allergic reaction, which may be caused by the combined action of CCDs and homologous proteins.

A relative quantitative comparison analysis of total glycans in eight plant glycoproteins revealed a link between differences in CCD content and the extent of the cross-allergic reaction. The higher was the CCD content, the more likely it was to cause extensive cross-allergic reactions. As shown in Fig. 4, the highest CCD content was in pollen, which was consistent with pollen-nut cross-allergic reactions (Vieths et al., 2002). Although the relative proportion of soybean glycoprotein CCDs was low, total glycan content of soybean glycoprotein was nevertheless 5–10 times greater than in other nuts and was second only to that in pollen. This finding is consistent with the reported soybean-nut cross-allergy (Branum and Lukacs, 2009). Kulis et al. found that mice allergic to cashew displayed a more severe allergic response if fed walnuts than if given cashews, which is consistent with the higher glycoprotein CCD content in walnuts compared to cashews (Kulis et al., 2009). Branum et al. found that hazelnut and almond displayed high cross-allergies

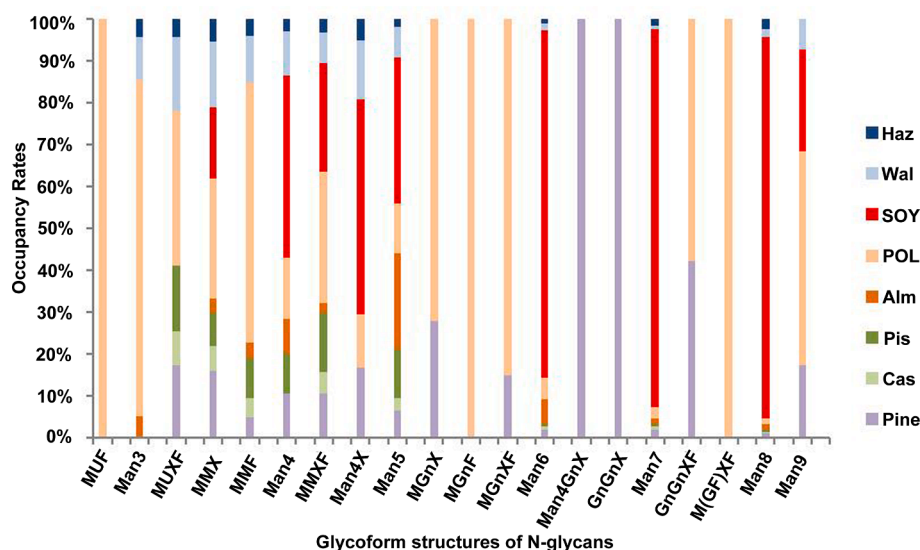


Fig. 2. Histogram showing the occupancy rates of each *N*-glycan in eight plant glycoproteins. Haz, hazelnut; Wal, walnut; SOY, soybean; POL, pollen; Alm, almond; Pis, pistachio; Cas, cashew; Pine, pine nut. M, mannose; F, fucose; X, xylose; U, uncore; Gn, *N*-acetylglucosamine; G, galactose.

Table 1

Quantitative comparison of *N*-glycans from eight plant glycoproteins. M, Mannose; F, Fucose; X, Xylose; U, uncore; Gn, *N*-acetylglucosamine; G, Galactose. Structural formulae: blue square, *N*-acetylglucosamine; orange stars, xylose; green circle, mannose; red triangle, fucose.

Monosaccharide composition	$m/z[M + Na]^+$	Speculative structure	Pine nut	Pistachio	Cashew	Walnut	Hazelnut	Almond	Soybean	Peach Pollen
MUF	917.33		0	0	0	0	0	0	0	11.94
Man3	933.33		0	0	0	1.85	0.40	0.89	0	10.93
MUXF	1049.33		2.61	2.38	1.22	2.66	0.65	0	0	5.59
MMX	1065.08		4.68	3.22	2.46	6.47	2.23	1.47	6.99	9.72
MMF	1079.33		2.56	4.89	2.42	5.81	2.17	2.04	0	32.57
Man4	1095.17		2.23	1.95	0	2.24	0.60	1.78	9.16	3.12
MMXF	1211.25		35.32	47.16	17.31	24.64	10.50	7.55	86.62	105.40
Man4X	1227.33		1.27	0	0	1.07	0.38	0	3.92	0.97
Man5	1257.08		5.15	9.25	2.39	5.91	1.44	18.54	27.91	8.93
MGnX	1268.33		1.10	0	0	0	0	0	0	1.59
MGnF	1282.45		0	0	0	0	0	0	0	8.74
MGnXF	1414.25		3.35	0	0	0	0	0	0	10.94
Man6	1419.33		2.73	0.63	1.47	2.49	1.58	9.17	128.70	7.32
Man4GnX	1430.33		0.66	0	0	0	0	0	0	0
GnGnX	1471.51		1.39	0	0	0	0	0	0	0
Man7	1581.33		2.99	1.04	1.45	1.37	2.50	2.29	151.46	3.19
GnGnXF	1617.33		1.74	0	0	0	0	0	0	3.28
M(GF)XF	1722.25		0	0	0	0	0	0	0	0.92
Man8	1743.17		1.22	0.70	0.46	2.55	3.22	1.88	124.46	1.07
Man9	1905.33		0.96	0	0	0.40	0	0	1.35	2.81

(Branum and Lukacs, 2009). Here, the relative proportions and structural types of CCDs differed between the two nuts, but their CCDs content was similar, indicating that content and structure of CCDs jointly affect the cross-allergic reaction. In this view, it would be inaccurate to regard CCDs as the sole indicators of cross-allergic reactions.

4. Conclusion

In this study, we provide for the first time a qualitative and quantitative comparison of *N*-glycans from eight plant-derived glycoproteins. Specifically, we report that the abundance of cashew and pistachio glycoprotein CCDs differs by only 1%; whereas that between cashew and walnut differs by 9%. Finally, almonds and hazelnuts glycoprotein CCDs

Table 2Analysis of the glycoform structure of *N*-glycans from eight plant glycoproteins.

Glycoproteins Type	<i>N</i> -glycans Type			
	Truncated	Complex	High mannose	Hybrid
Cashew	1	3	4	0
Pistachio	1	3	5	0
Walnut	1	3	7	1
Hazelnut	1	3	6	1
Almond	0	3	6	0
Soybean	0	2	6	1
Pine nut	1	7	6	2
Peach Pollen	2	8	7	1

also share a similar content. We speculate that the relative content of CCDs in different plant glycoproteins correlates positively with cross-allergic reactions between plants. For example, pollen had the highest total CCD content and is prone to cross-allergic reactions with soybeans and nuts. Pine nut glycoproteins contained a similarly high level of CCDs, so patients allergic to pine nuts should avoid eating walnuts, cashews, pistachios, hazelnuts, and soybeans, as well as avoid pollen. CCD content was the lowest in almond glycoproteins, implying that this nut does not cause serious cross-allergic reactions with other plants, and could be safely applied for immunotolerance therapy. At the same time, we report that pollen glycoproteins contain complex and rich CCDs, which can be used further to study the biological mechanism between plant glycoprotein CCDs and allergies.

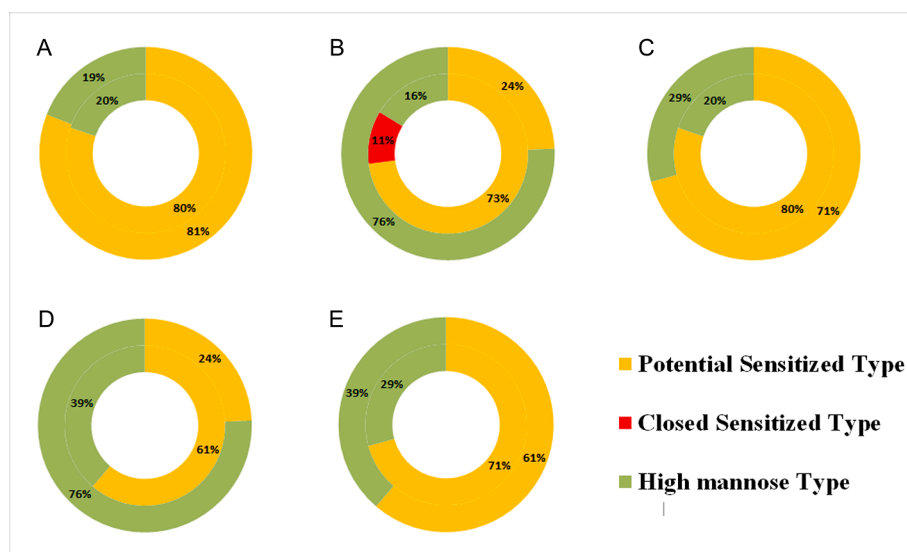


Fig. 3. Ring charts showing the proportion of *N*-glycan types in different plant glycoproteins. (A) Inner ring, cashew; outer ring, pistachio. (B) Inner ring, peach pollen; outer ring, almond. (C) Inner ring, cashew; outer ring, walnut. (D) Inner ring, hazelnut; outer ring, almond. (E) Inner ring, walnut; outer ring, hazelnut.

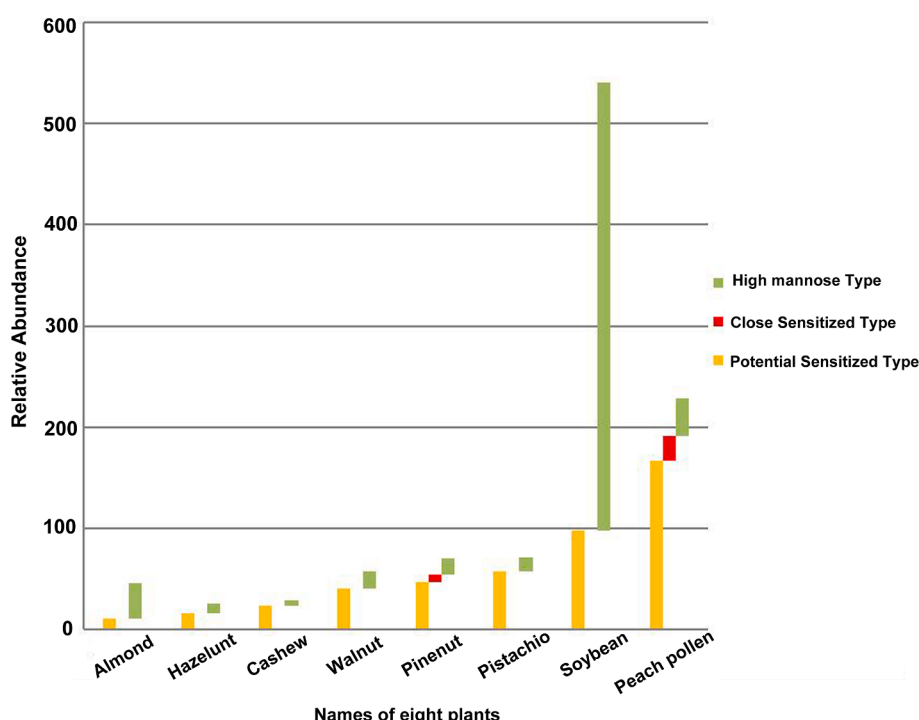


Fig. 4. Histogram showing the relative quantitative comparison of three types of *N*-glycans from eight plant glycoproteins.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodchem.2022.132440>.

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