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β(1,2)-Xylose and α(1,3)-Fucose Residues Have a Strong Contribution in IgE Binding to Plant Glycoallergens*

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Primary structures of the *N*-glycans of two major pollen allergens (Lol p 11 and Ole e 1) and a major peanut allergen (Ara h 1) were determined. Ole e 1 and Ara h 1 carried high mannose and complex *N*-glycans, whereas Lol p 11 carried only the complex. The complex structures all had a β(1,2)-xylose linked to the core mannose. Substitution of the proximal *N*-acetylglucosamine with an α(1,3)-fucose was observed on Lol p 11 and a minor fraction of Ole e 1 but not on Ara h 1. To elucidate the structural basis for IgE recognition of plant *N*-glycans, radioallergosorbent test analysis with protease digests of the three allergens and a panel of glycoproteins with known *N*-glycan structures was performed. It was demonstrated that both α(1,3)-fucose and β(1,2)-xylose are involved in IgE binding. Surprisingly, xylose-specific IgE antibodies that bound to Lol p 11 and bromelain did not recognize closely related xylose-containing structures on horseradish peroxidase, phytohemeagglutinin, Ole e 1, and Ara h 1. On Lol p 11 and bromelain, the core β-mannose is substituted with just an α(1,6)-mannose. On the other xylose-containing *N*-glycans, an additional α(1,3)-mannose is present. These observations indicate that IgE binding to xylose is sterically hampered by the presence of an α(1,3)-antenna.

In the early 1980s, it was reported for the first time that IgE antibodies in sera of pollen allergic patients can be directed to carbohydrate determinants on glycoproteins (1–3). The carbohydrate nature of these epitopes was supported by several characteristic properties, such as their periodate sensitivity and their resistance to heating and protease digestion. IgE antibodies directed to these carbohydrate structures were shown to be extremely cross-reactive not only between different plant-derived glycoproteins but also to glycoproteins from invertebrate animals (*e.g.* seafood and insect venoms) (2, 4–7). This high degree of cross-reactivity was explained by the conserved structure of *N*-glycans from plants and invertebrate animals, sharing several features that are not found in mammalian *N*-glycans (8). More recently, several research groups have confirmed the role of carbohydrate epitopes in IgE reactivity (9–22).

In plants, the *N*-glycosylation of proteins starts by the transfer of the oligosaccharide precursor $Glc_3Man_9GlcNAc_2$ in the endoplasmic reticulum (reviewed in Ref. 23). This structure can subsequently be modified by glycosidases and glycosyltransferases during transport of the glycoprotein through the endoplasmic reticulum, the Golgi apparatus, and the vacuole. Depending on the accessibility of the glycan side chain, these enzymes can convert the precursor to high mannose-type *N*-glycans ranging from $Man_9GlcNAc_2$ to $Man_5GlcNAc_2$ and then to complex-type *N*-glycans having an α(1,3)-fucose attached to the proximal glucosamine residue and/or a β(1,2)-xylose residue attached to the β-mannose. These linkages of fucose and xylose are typical for complex *N*-glycans from plants and invertebrate animals and are not found in mammals. More recently, it was demonstrated that complex *N*-glycans of plants can also carry Lewis a antennae (24, 25). When *N*-glycans are attached to a vacuolar glycoprotein, complex oligosaccharides can be further modified by the elimination of terminal residues (26). This last modification results in the formation of truncated oligosaccharide structures, called paucimannosidic-type *N*-glycans, having only α(1,3)-fucose and/or a β(1,2)-xylose residue linked to the core $Man_3GlcNAc_2$ or $Man_2GlcNAc_2$ (23). Both these α(1,3)-fucose and β(1,2)-xylose are (at least partly) responsible for the immunogenicity of plant-glycoproteins in mammals (8, 27–30). Horseradish peroxidase (HRP)[1] is a glycoprotein carrying *N*-glycans with both these monosaccharides (31). Polyclonal rabbit antiserum against HRP was shown to contain both anti-fucose and anti-xylose activity. Both activities could be separated into distinct antibody populations by affinity-chromatography with honeybee venom phospholipase A_2 (PLA_2) (32). PLA_2 carries an *N*-glycan with α(1,3)-fucose but without β(1,2)-xylose (33). An earlier study by Kurosaka *et al.*

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[1] The abbreviations used are: HRP, horseradish peroxidase; PLA_2, phospholipase A_2; RAST, radioallergosorbent test; BRO, bromelain; PHA, phytohemagglutinin; PAGE, polyacrylamide gel electrophoresis; ConA, concanavalin A; PNGase, peptide *N*-glycosidase; HPAEC-PAD, high pH anion exchange chromatography coupled to pulsed amperometric detection; MALDI-TOF, matrix-assisted laser desorption ionization-time of flight; ^{1}H NMR, proton nuclear magnetic resonance; BSA, bovine serum albumin; R_S, Spearman rank correlation coefficient.

(28) supports these observations by showing decreased binding of a goat antiserum against HRP after defucosylation or dexylosylation of the *N*-glycan of HRP. Tretter *et al.* (34) used defucosylation of PLA_2 to show the involvement of the α(1,3)-fucose in IgE binding. For β(1,2)-xylose, a role in IgE binding has also been suggested mainly on the basis of studies with zucchini-ascorbate oxidase (35). This glycoprotein was originally reported to be nonfucosylated (36), but recent studies have demonstrated that a significant fraction of its *N*-glycans carries both β(1,2)-xylose and α(1,3)-fucose (37).

Recently, Wilson and Altmann (38) analyzed the distribution of various complex and high mannose *N*-glycans in extracts of different mono- and dicotyledonous pollen. Grass pollens were found to contain predominantly β(1,2)-xylosylated/α(1,3)-fucosylated *N*-glycans but no high mannose structures. In contrast, tree pollen also contained high mannose *N*-glycans. The aim of this study was to determine the structures of three glycoallergen *N*-glycans and to investigate which structural elements are involved in IgE binding and consequently IgE cross-reactivity. To this end, we determined the primary structure of *N*-linked glycans from two purified major pollen allergens, Ole e 1 (olive tree) and Lol p 11 (rye grass) and of a purified major food allergen, Ara h 1 (peanut). IgE reactivity to these *N*-glycans was studied and compared with IgE reactivity to a number of glycoproteins with known primary structures.

EXPERIMENTAL PROCEDURES

Allergen Extracts and Purified Allergens—Grass pollen (*Dactylis glomerata* and *Lolium perenne*), birch pollen (*Betula verrucosa*), olive pollen (*Olea europaea*), and mugwort pollen (*Artemisia vulgaris*) were extracted as described previously (39). The major grass pollen allergen Lol p 11 was purified from *L. perenne* grass pollen extract by means of affinity chromatography using monoclonal antibody 2H4 (40). Ole e 1 was purified from *O. europaea* pollen extract by means of reversed-phase high pressure liquid chromatography as described earlier (12). Ara h 1 was purified from crude peanut extract by means of ion exchange chromatography as reported elsewhere (41).

Protein extracts from wild type *Arabidopsis thaliana* and a *cgl* mutant were made in 10 mM $NaHCO_3$ (10% w/v), pH 9.2, for 4 h. After filtration and centrifugation, the supernatants were dialyzed for 24 h against phosphate-buffered saline. For application in the radioallergosorbent test (RAST), the resulting extracts were coupled to CNBr-activated Sepharose 4B (2.5 mg of protein on 100 mg of Sepharose). The *cgl* mutant lacks *N*-acetylglucosaminyltransferase I activity and is unable to synthesize complex-type *N*-glycans, thereby accumulating $Man_5GlcNAc_2$ oligosaccharides (42).

Purified Glycoproteins and Neoglycopeptides—HRP, PLA_2 from bee venom, bromelain (BRO) from pineapple stem, and phytohemagglutinin-L (PHA) from red kidney bean were purchased from Sigma. PHA devoid of the α(1,3)-fucose residue was purified from a *mur1* mutant of *A. thaliana* expressing PHA.[2] This mutant is affected in the gene encoding for a GDP-D-mannose-4,6-dehydratase, an enzyme involved in the biosynthesis of L-fucose (43, 44).

BSA-Le^a/Le^x, obtained by coupling BSA with a 1:1 mixture of lacto-*N*-fucopentaose II (Galβ(1–3)[Fucα(1–4)]GlcNAcβ(1–3)Galβ(1–4)Glc) and lacto-*N*-fucopentaose III (Galβ(1–4)[Fucα(1–3)]GlcNAcβ(1–3)Galβ(1–4)Glc), was a generous gift from J.-C. Michalski (UMR 111, Lille, France).

Defucosylation of Bromelain Glycopeptides—Glycopeptides from bromelain were purified and subsequently defucosylated by mild trifluoroacetic acid treatment as described by Prenner *et al.* (29) and Tretter *et al.* (34, 45). Mass spectrometry analysis revealed <1% residual fucose content of defucosylated bromelain glycopeptides (BRO^{-fuc}). Control and defucosylated bromelain glycopeptide were coupled to Sepharose (1 μmol/100 mg) for application in RAST. Per test 5 nmol of Sepharose-coupled glycopeptide was used.

Protease Digestion—Proteinase K digestion of pollen extracts was carried out as described elsewhere (40). Briefly, pollen extract (25 mg/ml) was digested overnight at 50 °C with proteinase K (100 μg/ml). Digestion was stopped by heating the sample for 5 min at 100 °C. As controls, pollen extracts were also subjected to the same protocol in the absence of proteinase K. Digestion was monitored by measuring residual allergen activity with specific antibody reagents. The allergens tested were Bet v 1 (46) and profilin (47, 48) in the case of birch pollen, Dac g 1 and Dac g 5 for grass pollen (49), and profilin for mugwort pollen. Less than 1% of the control value was left in all cases. In addition, digest and control were analyzed by SDS-PAGE/silver staining. Digests did not show any protein bands (not shown). For application in RAST, digested extract (originating from 25 mg of dry weight) was coupled to 1 g of Sepharose.

Purified glycoproteins and allergens (100 μg of each) were digested with proteinase K (0.4 μg) and coupled to Sepharose (100 mg). Digestion was monitored by SDS-PAGE/silver staining. For Lolp 11, Ara h 1, Ole e 1, PLA_2, and bromelain no protein bands were detected after digestion. In contrast, HRP and PHA appeared unaffected by proteinase K treatment. Even at 100-fold higher protease concentrations no significant changes were observed on SDS-PAGE. In RAST analysis, treated (but unaffected as judged by SDS-PAGE) HRP and PHA were used.

Sera—From sera submitted for routine *in vitro* allergy diagnostics, a panel of 673 sera was selected on the basis of specific IgE antibodies directed to pollen and/or vegetable foods: pollen, >25 IU/ml; vegetable foods, >5 IU/ml. In most cases, a response to vegetable foods was accompanied by high titers of IgE against pollen.

SDS-PAGE/Immunoblotting—Purified Ole e 1, Lol p 11, and Ara h 1 were separated by SDS-PAGE on a 15% polyacrylamide gel under reducing conditions and were then transferred onto a nitrocellulose membrane. Immunodetections of β(1,2)-xylose- and α(1,3)-fucose-containing glycans were performed with specific IgG antibodies purified from a polyclonal rabbit antiserum against HRP as described previously (32). Affinodetection of high mannose-type *N*-glycans was carried out using concanavalin A (ConA) (50). ConA specifically binds to *N*-glycans containing 5–9 mannose residues.

Whole pollen extracts and their digests were separated by SDS-PAGE on 5–15% gradient gel. Gels were used for silver staining and immunoblotting. Immunoblots were probed with human sera for IgE binding. For detection of IgE, radiolabeled sheep antibodies against IgE were used.

Isolation of N-linked Glycans from the Allergens—*N*-linked glycans of Ole e1 and Ara h1 were released from the allergens by peptide *N*-glycosidase (PNGase) F (Roche Molecular Biochemicals) as previously reported (12). *N*-glycans of Lol p 11 were released by sequential digestions with pepsin (Sigma) and PNGase A (Roche Molecular Biochemicals) as previously reported (51, 52). The resulting free glycans were desalted on a Bio Gel P4 column (Bio-Rad) (40 × 1 cm) using water as eluent. Complex *N*-glycans were separated from the high mannose-type *N*-glycans by affinity chromatography on a concanavalin A-Sepharose 4B column (Amersham Pharmacia Biotech) as previously reported (53). For analysis by electrospray mass spectrometry, complex *N*-glycans from Ara h1 were conjugated with aminopyridine (54).

Analysis of N-linked Glycans—High pH anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) of oligosaccharides released from the allergens was carried out on a Dionex DX 500 system equipped with a GP 50 gradient pump and a CarboPac PA1 column. Oligosaccharides were separated using a linear gradient from 0 to 100 mM NaOAc in 100 mM NaOH at 1 ml/min over 30 min. High mannose-type *N*-glycans were identified by comparing their retention times to standard oligosaccharides isolated from RNase B (Man-5 and Man-6) and bean phaseolin (Man-7) as previously reported (52).

Electrospray Mass Ionization and Matrix-assisted Laser Desorption Ionization-Time of Flight (MALDI-TOF)—Electrospray mass ionization was performed on a quadrupolar mass spectrometer NERMAG R 1010U equipped with an Analytica of Branford atmospheric pressure electrospray source (Quad Servive, Poissy, France). The mass on charge (m/z) range was of 2000. The sample was dissolved in a 1:1 H_2O:CH_3OH solution containing 0.05% CH_3COOH and infused into the electrospray ion source at a flow-rate of 1.5 μl/min by a Harvard syringe pump (Harvard Apparatus). The ion source temperature was 80 °C (drying gas). Full scan spectra were acquired in the range of 500–1950 at a scan speed of 9 s.

MALDI-TOF mass spectra were measured on a Micromass (Manchester, UK) TofspecE matrix-assisted laser desorption ionization-time of flight mass spectrometer. This instrument was operated to an accelerating voltage of 25 kV in linear mode and to an accelerating voltage of 20 kV in reflector mode. The reflector had a potential of 26 kV. The apparatus was operated at a pressure of approximately 10^{-7} millibars in the source and 10^{-6} millibars in the analyser. The nitrogen laser wavelength was set at 337 nm having a pulse width of 4 ns. The MALDI-TOF mass spectra, performed in positive ion mode, were smoothed once and were performed in the reflector mode. The laser

[2] W.-D. Reiter, unpublished data.

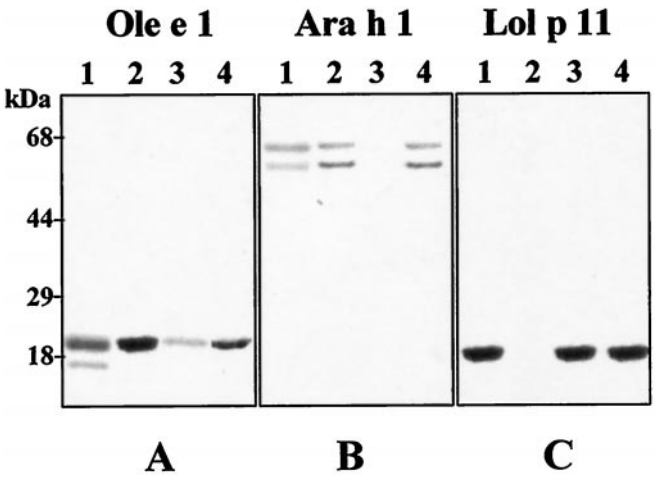


FIG. 1. **SDS-PAGE and Western blot analysis of allergens.** *A*, Ole e1; *B*, Ara h1; *C*, Lol p 11. *Lanes 1,* Coomassie Blue staining of the SDS-PAGE gel. *Lanes 2,* affinodetection with ConA. *Lanes 3,* immunodetection with antibodies specific for α(1,3)-fucose (anti-fucose antibodies). *Lanes 4,* immunodetection with antibodies specific for β(1,2)-xylose (anti-xylose antibodies).

shots were summed for each mass spectrum to have an acceptable signal to noise ratio. The solution containing the sample was prepared at a concentration of ~10 pmol μl^{-1} in water. Two to five μl of this solution were dissolved in the same volume of the matrix solution. The matrix solution was prepared by dissolution of 2 mg of 2,5-dihydroxybenzoïc acid in 200 μl of 70% acetonitrile in 0.1% trifluoroacetic acid. The sample matrix mixture obtained was homogenized, and 1 μl of this solution was deposited onto the sample holder and allowed to dry under vacuum.

Nuclear Magnetic Resonance (^{1}H NMR)—^{1}H NMR was performed at 400 MHz on a Bruker AMX400 spectrometer. The spectrum was recorded at 297 K in deuterium oxide (D_2O 99.97). Chemical shifts were expressed in ppm downfield from internal TMS and with an HOD presaturation sequence.

RAST and RAST Inhibition—RAST was performed as described previously (2). Briefly, serum (50 μl) was incubated overnight with 0.5 mg of Sepharose-coupled allergen in a final volume of 300 μl of phosphate-buffered saline, 0.3% BSA, 0.1% Tween-20. After washing away unbound serum components, radiolabeled sheep antibodies, directed to human IgE, were added. After overnight incubation and washing, bound radioactivity was measured. Results were expressed as international units/ml (IU/ml). Calculation was performed by means of a standard curve that was obtained by RAST with a dilution series of a chimeric monoclonal IgE antibody against the major housedust mite allergen Der p 2 and Sepharose-coupled mite extract (55). A result of >0.3 IU/ml was regarded as positive. One IU is 2.4 ng of IgE.

For RAST inhibition, serum was preincubated for 1 h with (serial dilutions of) inhibitor prior to the addition of Sepharose-coupled allergen. For the uninhibited value, serum was preincubated with phosphate-buffered saline, 0.3% BSA, 0.1% Tween-20. Subsequent steps were identical to those described for the RAST.

Statistical Analysis—Correlations between IgE responses were analyzed by Spearman rank correlation. Statistical significance was accepted at $p < 0.05$.

RESULTS

N-Glycosylation Analysis of Ole e 1, Ara h 1, and Lol p 11

Western Blot Analysis of the N-Glycosylation of Allergens—A preliminary analysis of the *N*-linked glycans linked to Ole e1, Ara h1, and Lol p 11 was obtained by affino- and immunodetection on blots using glycan-specific probes (Fig. 1). The major Ole e 1 band was found to contain both high mannose- and complex-type *N*-glycans, the latter with both fucose and xylose (Fig. 1*A*). A band with slightly lower molecular weight was not detected by glycan-specific reagents, confirming its nonglycosylated nature. The two isoforms of Ara h1 were detected by ConA and anti-xylose antibodies but not by anti-fucose antibodies, indicating that this allergen is *N*-glycosylated by both high mannose-type and nonfucosylated complex *N*-glycans. Lol p 11 was only recognized by both antibody reagents directed to complex *N*-glycans (Fig. 1*C*).

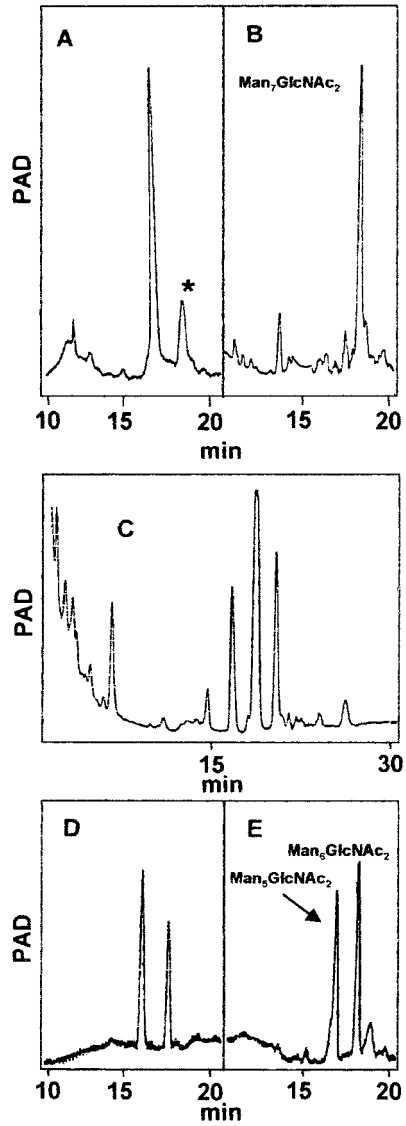


FIG. 2. **HPAEC-PAD chromatography.** HPAEC-PAD chromatography of oligosaccharides released from Ole e1 and Ara h 1 by PNGase F and separated by affinity chromatography on a ConA-Sepharose column. *A,* nonretained fraction from Ole e 1; *B,* retained fraction from Ole e 1. *, minor nonidentified *N*-glycan. *C,* profile of total *N*-glycans from Ara h 1; *D,* profile of *N*-glycans from Ara h 1 nonretained by affinity chromatography on a ConA-Sepharose column. *E,* profile of *N*-glycans from Ara h 1 retained by affinity on ConA. $Man_5GlcNAc_2$ and $Man_6GlcNAc_2$, high mannose-type *N*-glycans identified by comparison of the retention times with standard oligosaccharides purified from ribonuclease B.

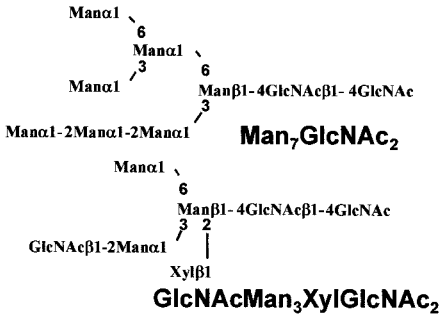
Structure of N-Glycans Isolated from Ole e1—Ole e1 was deglycosylated by PNGase F. SDS-PAGE analysis of the digest indicated that the allergen was not completely deglycosylated (not shown). This can be explained by the presence on Ole e 1 of a minor glycoform carrying an α(1,3)-fucose-containing *N*-glycan (Fig. 1*A*, *lane 3*). This type of *N*-glycans is resistant to deglycosylation by PNGase F (45). The structure of this α(1,3)-fucose *N*-glycans was not further investigated. The major *N*-glycans released by PNGase F were then separated into high mannose-type *N*-glycans and complex *N*-glycans on a ConA-Sepharose column. The retained fraction was eluted with α-methyl mannoside. As shown in Fig. 2, the analysis of the nonretained (Fig. 2*A*) and retained fractions (Fig. 2*B*) by

TABLE I

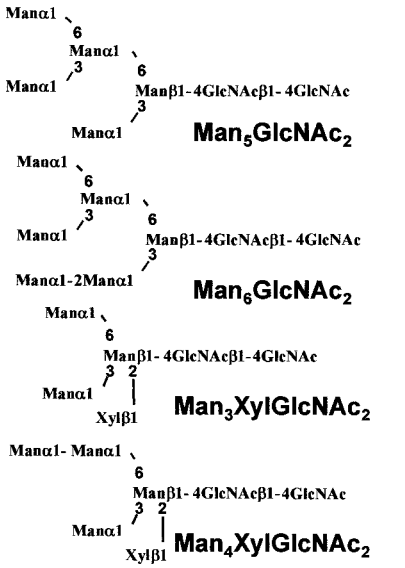
N-Glycan structures of glycoallergens

Structures of the major *N*-linked glycans isolated from Ole e1, Ara h1, and Lol p 11. Exact linkages between the terminal mannose residue and the core of $Man_4XylGlcNAc_2$ and $Man_2XylFucGlcNAc_2$ oligosaccharides were not determined. Minor α(1,3)-fucosylated N-glycans were also detected in Ole e 1.

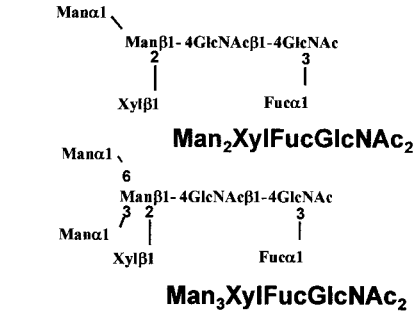
Ole e1



Ara h1



Lol p11



HPAEC-PAD indicated that one major oligosaccharide is present in both fractions. The major high mannose-type *N*-glycan was identified as $Man_7GlcNAc_2$ on the basis of a similar retention time as the high mannose structure released from phaseolin. The structure of this oligosaccharide was confirmed by comparison of its proton NMR spectrum with data reported in the literature (56). Using this comparative NMR approach, the structure of the oligosaccharide detected in the nonretained fraction was identified as $GlcNAcMan_3XylGlcNAc_2$. Structures of both major *N*-glycans of Ole e 1 are shown in Table I.

Structure of N-Glycans Isolated from Ara h1—Because no α(1,3)-fucose residues were detected on blot with specific anti-fucose antibodies (Fig. 1*B*, *lane 3*), the *N*-glycans from Ara h1 were completely released by PNGase F and then analyzed by HPAEC-PAD. Four *N*-glycans were detected (Fig. 2*C*). These glycans were separated on a ConA-Sepharose column. Two *N*-glycans were retained on the column and were identified as $Man_5GlcNAc_2$ and $Man_6GlcNAc_2$ by comparison with standards prepared from ribonuclease B (Fig. 2*E*) (52). The two oligosaccharides in the nonretained fraction (Fig. 2*D*) were conjugated with aminopyridine and analyzed by electrospray mass spectrometry. Two $(M + Na)^+$ molecular ions at m/z 1121 and 1283 were detected and assigned respectively to the pyridylamino derivatives of the xylosyl-containing *N*-glycans $Man_3XylGlcNAc_2$ and $Man_4XylGlcNAc_2$. In the latter structure, the linkage of the additional mannose residue on the α(1,6)-mannose arm of the core $Man_3GlcNAc_2$ was not determined. Structures of the four glycans on Ara h 1 are shown in Table I.

Structure of N-Glycans Isolated from Lol p 11—Because Lol p 11 was detected on blot with comparable density by anti-fucose as by anti-xylose antibodies (Fig. 1*C*), this allergen was supposed to be predominantly *N*-glycosylated by complex *N*-glycans containing both α(1,3)-fucose and β(1,2)-xylose. These *N*-glycans were released from the allergen by successive digestions with pepsin and PNGase A. In contrast to PNGase F, this *N*-glycosidase is able to release all *N*-linked glycans, including those carrying an α(1,3)-fucose linked to the proximal glucosamine (57). Two major *N*-glycans were detected by HPAEC-PAD chromatography (not shown). The mixture of *N*-glycans was directly analyzed by MALDI-TOF. As illustrated in Fig. 3, two main $(M + Na)^+$ molecular ions at m/z 1049 and 1211 were detected and assigned to $Man_2XylFucGlcNAc_2$ and $Man_3XylFucGlcNAc_2$. The linkage between the terminal mannose residue and the β-mannose of the core of the $Man_2XylFucGlcNAc_2$ structure was not determined. Minor N-linked oligosaccharides were also detected in the MALDI-TOF spectrum and could be assigned, on the basis of the mass values, to $Man_2FucGlcNAc_2$ (m/z 917) and $GlcNAcMan_3XylFucGlcNAc_2$ (m/z 1414).

Characterization of IgE Antibody Responses Against the N-Glycans

IgE Antibodies against Proteinase K-digested Pollen Extracts—A panel of 673 sera was screened for carbohydrate-specific IgE by RAST with proteinase K-digested extracts of grass (*D. glomerata*), birch (*B. verrucosa*), and mugwort (*A. vulgaris*) pollen. A positive RAST (>0.3 IU/ml) was observed in 55.7, 82.8, and 50.7% of the cases, respectively. The majority of these responses was, however, extremely low (<1.0 IU/ml). A total of 212 sera had >1.0 IU/ml against one or more of the pollen digests: 87 for *D. glomerata*, 166 for *B. verrucosa,* and 67

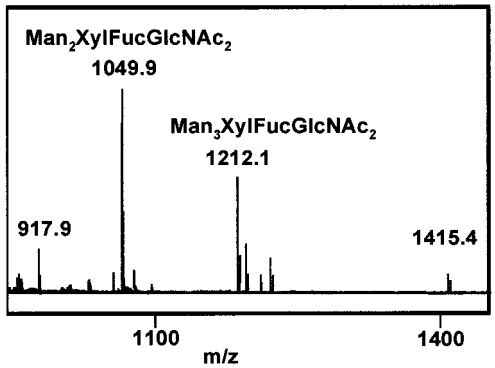


FIG. 3. **MALDI-TOF analysis.** MALDI-TOF mass spectrum of *N*-glycans isolated from Lol p 11 by PNGase A digestion. Major $(M + Na)^+$ molecular ions were assigned to $Man_2XylFucGlcNAc_2$ and $Man_3XylFucGlcNAc_2$.

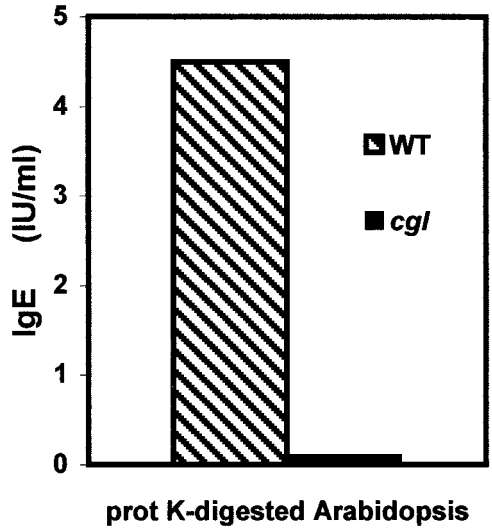


FIG. 4. **Effect of *cgl*-mutation on IgE binding.** IgE binding to wild type *A. thalania* extract and an extract of a *cgl* mutant. Results are expressed in IU/ml of specific IgE. *WT*, wild type.

for *A. vulgaris*. Spearman rank correlation of these IgE responses revealed no significant correlations between IgE responses to birch pollen digest and the other two pollen digests ($R_S < 0.1$; $p > 0.5$). Only the responses to grass pollen and mugwort pollen digests showed a weak correlation: R_S, 0.37; $p < 0.0001$. This lack of correlation was unexpected on the basis of the reported cross-reactivity of sugar-reactive IgE. In many cases, sera were selectively reactive with one or two of the pollen digests. This was observed for all three pollen species and in different combinations. To exclude that discrepancies were simply caused by IgE reactivity with different protease-resistant proteins, some sera were analyzed on immunoblot with undigested and digested pollen extracts (not shown). All sera tested demonstrated clear IgE binding to undigested pollen extracts but none bound to digested pollen extracts, indicating that no protease resistant peptides roughly between 5 and 100 kDa can explain the binding to digested pollen extracts.

Effect of cgl Mutation on IgE Binding to N-Glycans—To investigate the role in IgE binding of α(1,3)-fucose/β(1,2)-xylose containing *N*-glycans, RAST analysis was performed with wild type *Arabidopsis thalania* and a *cgl* mutant. The wild type produces both complex and high mannose *N*-glycans, whereas the mutant only produces high mannose *N*-glycans because it lacks *N*-acetylglucosaminyltransferase I activity. IgE binding to protease-digested extract of this mutant strain was undetectable, whereas the wild type control was clearly positive (Fig. 4). This confirms the pivotal role of complex plant *N*-glycans in IgE binding.

IgE Responses Against Digests of Ole e 1, Ara h 1, and Lol p 11—Sixty-six of the sera with > 1.0 IU/ml IgE against pollen digests were selected for RASTs with digests of Ole e 1, Ara h 1, and Lol p 11. The selection was largely on the grounds of availability but turned out to be random with respect to the IgE reactivity with the three pollen digests. Fourteen sera had a negative RAST for all three purified allergen digests. Only 9/66 sera had a positive RAST for Ara h 1 digest (Table II). For Ole e 1 and Lol p 11, these figures were 34/66 and 37/66, respectively (Fig. 5*A*). No significant correlation of IgE responses to both pollen-allergen digests was found (R_S, 0.11; *p,* 0.5).

Subsequently, sera were tested by RAST on a panel of digests of purified glycoproteins with known *N*-glycan structures: BRO (58), HRP (31), PHA (59), and PLA_2 (33) (Table III). To test a non-α(1,3)-fucosylated plant glycoprotein, PHA was expressed in the *mur 1* mutant of *A. thalania* and characterized.[2] This lectin, designated as PHA^{-fuc}, was found to be *N*-glycosylated by $Man_3XylGlcNAc_2$. Only about 1–2% of L-Gal was detected in place of L-Fuc, similar to what was described for the mutant *Arabidopsis* (43, 44).

TABLE II
Glycoallergen digests RAST

IgE responses of Ara h 1 positive sera to the three allergen digests. Results are expressed in IU/ml of specific IgE. NEG, ≤0.3 IU/ml.

Serum no.	Ara h 1	Ole e 1	Lol p 11
168	0.8	3.1	NEG
326	2.4	0.4	NEG
392	6.5	NEG	NEG
716	0.5	2.2	4.9
730	0.8	0.4	0.4
806	0.3	0.7	4.9
831	4.5	0.7	NEG
849	12.0	1.0	3.6
879	0.4	0.4	0.4

IgE reactivity to Ole e 1 digest did not show significant correlations with any of the glycoproteins tested. The same was true for IgE reactivity to Ara h 1. IgE reactivity to BRO correlated very well (R_S, 0.91; $p < 0.00001$) with Lol p 11 (Fig. 5*B*). Unexpectedly, this was not the case for HRP carrying *N*-glycans very similar to BRO and Lol p 11 (Fig. 5*C*) but with a higher degree of substitution of the core mannose (R_S, 0.12; *p,* 0.4). IgE reactivity to HRP showed a better correlation with that to PLA_2 and PHA (Fig. 5, *D* and *E*), with a R_S of 0.51 (*p,* 0.0005) and 0.66 ($p < 0.00001$), respectively. Twenty-one sera with a positive RAST for PHA were tested for IgE antibodies against transgenic PHA^{-fuc}. Only 8 of 21 still recognized PHA without α(1,3)-fucose but with β(1,2)-xylose (Fig. 5*F*). These sera were almost without exception positive on xylose containing *N*-glycans from HRP, BRO, Lol p 11, and Ole e 1 (Table IV). The importance of the β(1,2)-xylose in IgE recognition was further supported by the comparison of IgE binding to PLA_2 and Lol p 11. Thirteen of 37 sera reactive with Lol p 11 did not recognize PLA_2 (Fig. 5*G*); the main structural difference between their *N*-glycans being the lack of xylose in PLA_2. Finally, purified glycopeptide of BRO was defucosylated by mild trifluoroacetic acid treatment. Defucosylation did not affect the RAST of sera ($n = 4$) suspected to be xylose-specific (*i.e.* BRO-positive but PLA_2-negative) but unreactive with HRP and PHA/PHA^{-fuc} (not shown). In contrast, sera ($n = 4$) with IgE antibodies predominantly directed to fucose (*i.e.* similar reactivity to BRO and PLA_2) lost most of their reactivity upon defucosylation (not shown). The IgE binding capacity of BRO^{-fuc} decreased by approximately a factor 10 for these

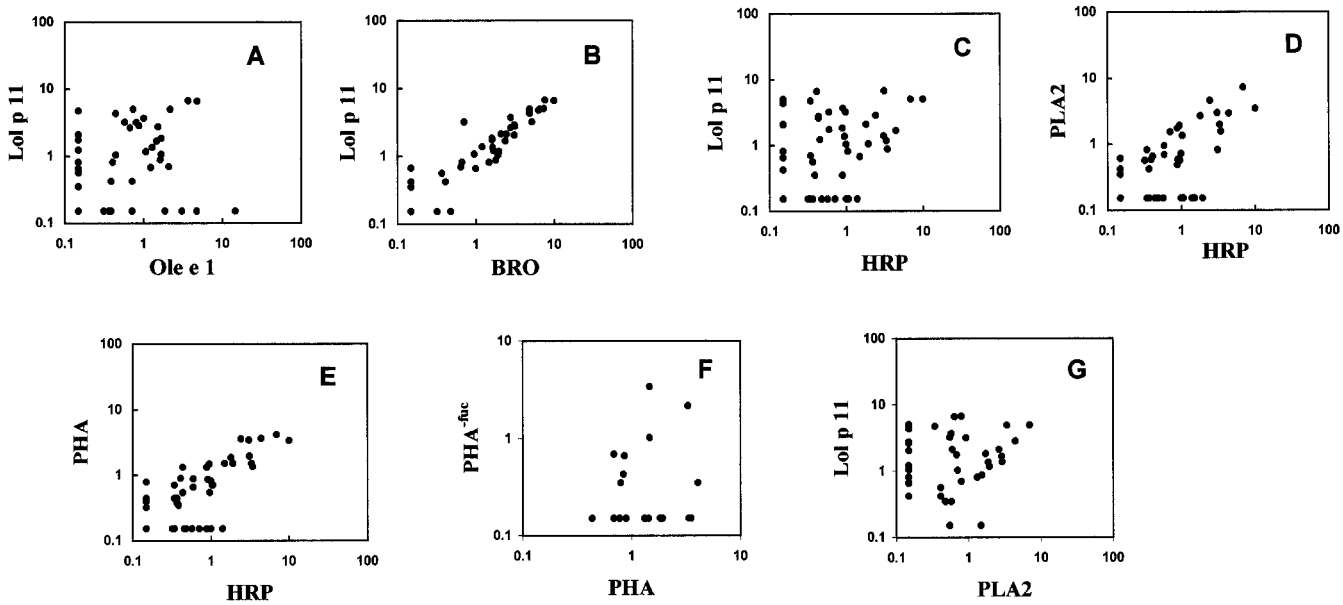
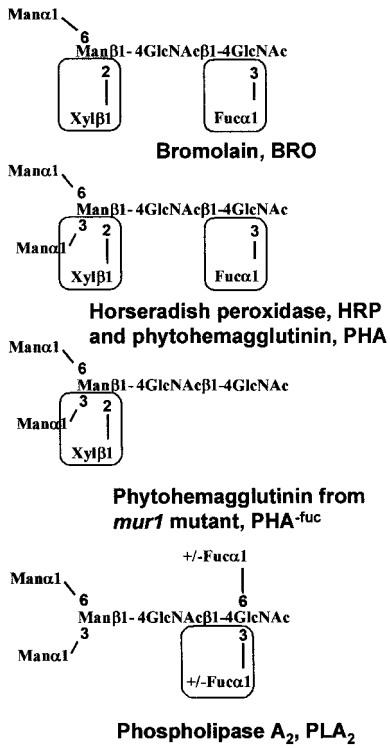


FIG. 5. **RAST analysis.** Correlation of IgE responses to *N*-glycans from different glycoproteins. RAST was performed with Sepharose-coupled proteinase K digests of glycoproteins. Results are expressed in IU/ml of IgE.

TABLE III
N-glycan structures of glycoproteins

Structures of the major N-linked glycans from BRO, HRP, PHA, PHA^{-fuc}, and PLA_2 are shown. Typical plant (and invertebrate animal) substitutions are framed.



sera, whereas no change was observed for xylose-specific sera (Fig. 6).

Are Lewis Motifs Involved in IgE Binding?—To investigate whether the Le^a epitope recently identified in plants is involved in IgE binding, RAST was performed with BSA conjugated with both Le^a and Le^x. No involvement of Le^a (or Le^x) in IgE recognition of plant *N*-glycans was found. Only 1 of 31 sera (from the original panel of 66 sera) tested had specific IgE against this neoglycopeptide. Further analysis showed that the positive RAST was caused by IgE binding to BSA.

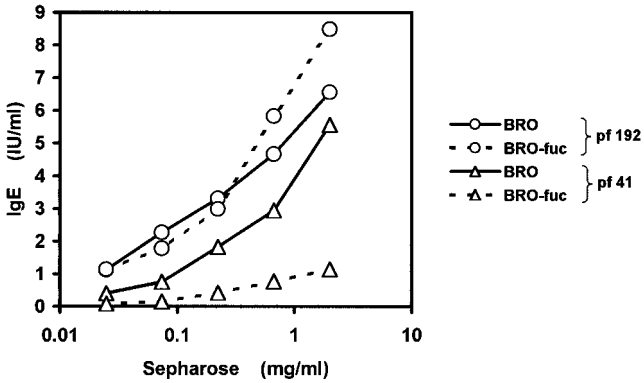
DISCUSSION

IgE cross-reactivity between pollen and vegetable foods has been explained largely by the presence of homologous proteins in both allergen sources (60). The best known examples are a pathogenesis-related protein from birch pollen (Bet v 1) and its homologues in fruits and nuts (46, 61, 62) and the ubiquitous protein profilin (group 2 allergen from tree pollen or group 12 allergen from grass pollen) that is present in all eukaryotic cells (48, 62). IgE cross-reactivity based on conserved plant *N*-glycans has attracted far less attention. Only for two major inhalant allergens primary structures of *N*-glycans were reported: Cry j 1 from Japanese cedar pollen (63) and BG60 from pollen of the subtropical grass *Cynodon dactylon* (17). In these studies very little or no attention was given to IgE binding characteristics. To our knowledge, so far no structural data were reported for *N*-glycans on major food allergens. Our study has for the first time produced structural data on *N*-glycans from a major allergen of a temperate grass species, a nonconiferous tree and a food, in combination with their IgE binding characteristics.

From both affino- and immunodetection on blots with glycan-specific probes and structural analysis by chromatography, NMR, and mass spectrometry, the different major *N*-linked oligosaccharides were identified. Ole e1 was found to have one major complex *N*-glycan, $GlcNAcMan_3XylGlcNAc_2$, and one major high mannose *N*-glycan, $Man_7GlcNAc_2$. A minor complex *N*-glycan having an $\alpha(1,3)$-fucose residue attached to the proximal glucosamine residue was detected on blot but not structurally identified. These structures were also recently reported for a crude olive pollen extract (38). In the extract, however, the most abundant complex *N*-glycan had two terminal *N*-acetylglucosamines instead of one. Compared with the structures that were determined on the major tree pollen allergen, Cry j 1, Ole e 1 was poor in fucose (63). Both high mannose ($Man_5GlcNAc_2$ and $Man_6GlcNAc_2$) and nonfucosylated complex *N*-glycans ($Man_4XylGlcNAc_2$ and $Man_3XylGlcNAc_2$) were identified in Ara h 1. In contrast, Lol p 11 is *N*-glycosylated exclusively by fucose/xylose-containing complex *N*-glycans ($Man_2XylFucGlcNAc_2$ and $Man_3XylFucGlcNAc_2$). The lack of

TABLE IV
Glycoprotein digests RAST
IgE responses of PHA^{-fuc} positive sera. Results are expressed in IU/ml of specific IgE. NEG, ≤0.3 IU/ml.

Serum no.	PHA^{-fuc}	BRO	HRP	Lol p 11	Ole e 1	Ara h 1
414	0.7	5.2	0.6	3.2	0.8	NEG
713	3.4	1.0	1.9	1.0	1.7	NEG
716	2.1	6.6	10.1	4.9	2.2	0.5
765	1.0	NEG	1.5	0.7	1.2	NEG
806	0.3	4.9	6.9	4.9	0.7	NEG
831	0.7	NEG	1.1	NEG	0.7	4.5
836	0.3	1.9	1.0	1.0	0.4	NEG
849	0.4	2.8	0.9	3.6	1.0	12.0



	HRP	PHA	PHA^{-FUC}	PLA_2
pf 192	< 0.3	0.3	< 0.3	< 0.3
pf 41	3.1	3.4	< 0.3	2.9

FIG. 6. **Defucosylation of bromelain glycopeptides.** The influence of defucosylation on IgE binding was analyzed by RAST. Sepharose-coupled bromelain glycopeptides (defucosylated and control) were titrated and assayed with a serum with preference for xylose (*pf 192*) and a serum with preference for fucose (*pf 41*), respectively. IgE binding of pf 192 was not affected by defucosylation. pf 41 showed at least a 10-fold decrease in IgE binding. For both sera, IgE titers (in IU/ml) against glycans from HRP, PHA, PHA^{-fuc}, and PLA_2 are shown below the graph.

high mannose structures is in agreement with the study by Wilson and Altmann (38) with pollen extracts. Compared with the structures reported for the Bermuda grass allergen BG60, there is a marked difference. This subtropical allergen carried predominantly nonxylosylated complex *N*-glycans (17).

IgE reactivity to protease-resistant structures is a very common feature in pollen-allergic patients (5). In at least 50% of all sera tested, IgE antibodies against proteinase K-digested pollen extracts were detected. Although involvement of protease-resistant proteins can not be ruled out completely (64), immunoblot analysis revealed no IgE-binding proteins at all. Unexpectedly, IgE responses against digests of grass, tree, and weed pollen demonstrated only low or no significant correlation. In other words, this study has demonstrated that carbohydrate-mediated IgE reactivity can not simply be explained by the presence of highly conserved *N*-glycan structures with fucose, xylose, or a combination of both. If this simple scheme was valid, a better correlation would have been found between IgE reactivity to *e.g.* grass pollen digest and birch pollen digest, both with >60% of their *N*-glycans being xylosylated and fucosylated (38). Therefore, other factors have to be involved besides the mere presence of both monosaccharides.

A significant group of sera had IgE antibodies that selectively reacted with only one of the pollen digests. The majority of these sera did not, however, show IgE reactivity to any of the digests of the purified allergens and glycoproteins. It is, therefore, unlikely that these sera recognize similar types of complex *N*-glycans as present on these glycoproteins. Alternatively, high mannose structures could be involved in IgE binding. This is unlikely from an immunologic perspective because these structures are not unique to plants but are ubiquitous in mammals as well. High mannose *N*-glycans were reported to be absent in grass pollen extracts in contrast to birch pollen extract (38). Sera with IgE antibodies to birch but not to grass pollen digest were, however, almost without exception negative to the high mannose carrying glycoproteins Ara h 1, Ole e 1, and PHA. Moreover, IgE reactivity was virtually absent to digest of the *cgl* mutant of *Arabidopsis* that can only produce high mannose *N*-glycans. All together, involvement of high mannose structures in IgE binding is unlikely. The observed lack of IgE reactivity with BSA-Le^a/Le^x excludes Le^a antennae, recently reported in plants (24, 25), as a possible explanation for the observed discrepancies. An alternative explanation could be IgE reactivity to *O*-linked glycans. This has been described for allergens from invertebrate animals (65) but so far not for plants. Currently, IgE reactivity to *O*-linked glycans on plant glycoproteins is under investigation.

Characterization of IgE responses against *N*-glycans using purified glycoproteins turned out to be more problematic than we originally thought. This is most likely caused by the variability of glycosylation observed in plant glycoproteins. A single *N*-glycosylation site usually carries a large variety of structurally different but closely related carbohydrate structures (microheterogeneity). With time the number of identified *N*-glycans/site increases. This is clearly illustrated by reports on HRP (31, 66, 67) and PLA_2 (33, 68). Frequently, there are major and minor forms/site, and this will largely determine their immunological relevance. Nevertheless, a minor form could have relevance for IgE binding. Both Ara h 1 and Ole e 1 carry nonfucosylated complex *N*-glycans, but their IgE reactivity is completely different. Part of the explanation for this discrepancy could be the presence of a minor fucosylated glycan on Ole e 1, that is absent on Ara h 1.

Another problem that was encountered is that the presence of a xylose was not always a "guarantee" for IgE recognition. Recognition of the xylose appears to be dependent on the degree of substitution of the core mannose influencing the conformation of the epitope. In bromelain, only an α(1,6)-linked mannose is present next to the β(1,2)-xylose (58). In Lol p 11, this type of complex *N*-glycan is of major importance as well. This simple form has not been reported for any of the other glycoproteins tested (31, 33, 59, 66–68). Despite the presence of a xylose in HRP, its IgE reactivity correlates better with the nonxylosylated PLA_2 (33, 68) than with bromelain or Lol p 11. The most likely explanation of this phenomenon is that additional substitutions to the core mannose sterically hamper IgE binding to the xylose-containing epitope. To exclude that fucose reactivity played a role in the observed differences, bromelain glycopeptide was defucosylated. This allowed comparison between two xylosylated glycans only differing in their degree of substitution of the core mannose, BRO^{-fuc} and PHA^{-fuc}. RAST analysis demonstrated that BRO^{-fuc} was still recognized by IgE from

sera that were unreactive with PHA^{-fuc}, thereby supporting the hypothesis that the presence of the α(1,3)-mannose in PHA^{-fuc} prevented IgE recognition. Finally, another potentially complexing factor is the interaction between the monosaccharide residues of the *N*-glycan and the peptide backbone. Differences in adjacent peptide sequences can have different effects on the structure and mobility of the *N*-glycan. For bromelain it was reported that mobility of its *N*-glycan is increased in a small glycopeptide compared with the native glycoprotein (69). Because HRP and PHA were found to be very resistant to proteinase K digestion, this could have negatively influenced the mobility of their glycan structures and thereby their IgE binding capacity, compared with those glycoproteins that were readily digested.

Despite all these complexing factors, this study has established the postulated role (35) for β(1,2)-xylose in IgE binding and confirmed the involvement of α(1,3)-fucose (34). These conclusions are largely based upon the comparison of the IgE binding characteristics of PHA *versus* PHA^{-fuc} and of Lol p 11 and bromelain *versus* PLA_2. The primary structures that were elucidated of the *N*-glycans on two major inhalant allergens and a major food allergen are an important prerequisite for further studies into IgE binding carbohydrate epitopes and their role in pollen-vegetable food cross-reactivity.

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