

# Glycoproteins are species-specific markers and major IgE reactants in grass pollens

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## Summary

Grass pollen allergic patients are concomitantly exposed and sensitized to pollens from multiple Pooideae (i.e. common grass) species. As such, they are currently desensitized by allergen-specific immunotherapy using extracts made from mixes of pollens from *Anthoxanthum odoratum*, *Dactylis glomerata*, *Lolium perenne*, *Phleum pratense* and *Poa pratensis*. Herein, we demonstrate that species-specific glycoprotein patterns are documented by 1D and 2D electrophoresis and Western blotting analysis, which can be used as an identity test for such pollens. Most allergens are glycoproteins bearing complex *N*-glycans encompassing  $\beta$ 1,2 xylose and  $\alpha$ 1,3 fucose glycoepitopes. Glycoepitope destruction using periodate oxidation has no impact on seric IgE reactivity in 75% atopic patients ( $n = 24$ ). The latter have thus no significant IgE responses to carbohydrate-containing epitopes. In contrast, periodate treatment strongly impairs IgE recognition of glycoallergens in 25% of patients tested, demonstrating the presence of carbohydrate-specific IgE in those patients. While the clinical impact of carbohydrate-specific IgE is still a matter of controversy, the presence of these IgE in the serum of many allergic patients illustrates the need for cross-reacting carbohydrate epitope-free recombinant allergens to develop relevant diagnostic tests. These data also support the pertinence of mixing multiple grass pollens to desensitize atopic patients, with the aim to broaden the repertoire of glycoepitopes in the vaccine, thus mimicking natural exposure conditions.

**Keywords:** allergy, grass pollen, glycoepitope, glycoproteome, glycan-specific IgE, cross-reactive carbohydrate determinant.

## Introduction

The number of allergic patients has strongly increased over the past few decades, and allergic diseases currently concern more than 25% of the European population. Pollinosis affects 50% of allergic patients (Corti *et al.*, 2005), and grass pollens are one of the most important airborne allergen sources worldwide. Allergy diagnosis and treatment require well-characterized allergens, and the diagnosis of grass pollen allergy has greatly improved owing to the availability of purified recombinant allergens. Many allergens are glycosylated, and the role of carbohydrates in initiation and exacerbation of allergic responses through the induction of glycan-specific IgE antibodies is established (Okano *et al.*, 2001; Soldatova *et al.*, 2007; Seppälä *et al.*, 2009). The presence of an oligosaccharide side chain attached to the protein backbone impacts how the allergen stimulates immune cells (Shreffler *et al.*, 2006) and, as an example, non-mammalian carbohydrate structures have recently been shown to act as Th2 adjuvants on monocyte-derived dendritic cells. The presence of IgE specific for *N*-glycans in sera from allergic patients also explains the cross-reactivity observed *in vitro* for a wide variety of plant and hymenoptera venom glycoallergens bearing a common  $\alpha$ 1,3 fucose-containing glycoepitope (Gomord *et al.*, 2010). While the impact of these cross-reacting carbohydrate epitopes (CCD) at the surface of some allergens is obvious for allergy diagnosis, their clinical impact is still a matter of controversy (Malandain, 2005; Altmann, 2007).

Therapeutic vaccines used for allergic patients' desensitization are produced from aqueous extracts of natural source materials such as grass pollens or house dust mites. In case of grass pollen allergy desensitization, a cocktail of five grass pollen protein extracts made from *Anthoxanthum odoratum*, *D. glomerata*, *Lolium perenne*, *Phleum pratense* and *Poa pratensis* is widely used (Moingeon *et al.*, 2008). In this study, in the prospect of a qualitative and quantitative standardization and to improve the consistency of the different batches of pollen extracts, we developed a proteomic approach to identify markers specific for each pollen species constitutive of the desensitization mix. Markers specific for each grass species pollen were identified in their glycoproteomes analysed using either 1D or 2D gel electrophoresis. In addition, our study sheds further light on the contribution of carbohydrate-based epitopes to IgE responses in grass pollen allergic patients, with evidence that the IgE reactivity pattern partly or eventually fully overlap the glycoproteome pattern.

## Results and discussion

### Grass pollens show species-specific glycoprotein patterns after SDS-PAGE

Complex allergen extracts are commonly used in diagnostic tests as well as in immunotherapy, but their preparations are difficult to standardize and their composition may vary from batch to batch. To define a simple method allowing a fast identification of

grass pollens used for desensitization, we compared their specific protein and glycoprotein patterns after SDS–PAGE. Proteomes of pollens from five common allergenic grass species in Europe are presented in Figure 1a, after Coomassie blue staining of SDS–PAGE gels. These protein patterns are too complex to distinguish the various grass species. In contrast, glycoprotein patterns are simpler and show some glycoprotein markers specific for each pollen species (Figure 1b,c). This is illustrated for instance after affindetection of glycoproteins containing high-mannose-type *N*-glycans with concanavalin A (Con A), a lectin that binds specifically to internal and non-reducing  $\alpha$ -mannose residues in *N*-glycans (Figure 1b). While most glycoproteins detected in Figure 1b have a molecular mass comprised between 50 and 100 kDa, each glycoprotein pattern contains species-specific glycoproteins, for example a double band at 75 kDa for ryegrass (Lp), or 44 and 46 kDa proteins for orchard grass (Dg) or sweet vernal grass (Ao), respectively. The presence of these specific markers was confirmed in six independent gel electrophoresis analyses. Species-specific glycoproteomes were further observed after immunodetection of glycoproteins with complex *N*-glycans containing  $\beta$ 1,2 xylose- and  $\alpha$ 1,3 fucose glycoepitopes (Figure 1c). Under such conditions, a high number of glycoprotein species with molecular masses ranging from 20 to 100 kDa were immunodetected in each pollen extract, with patterns typical for each grass species. Some grass pollen proteins are also glycosylated with complex *N*-glycans containing Lewis a ( $Le^a$ ) antennae. This Gal $\alpha$ 1,3(Fuc $\alpha$ 1,4)GlcNAc sequence is usually found on cell surface glycoconjugates in mammals and is involved in cell–cell recognition and cell adhesion processes. The plant  $Le^a$ , first described by Fitchette-Lainé *et al.* (1997) and Melo *et al.* (1997) in the plant complex *N*-glycans, is a good marker of extracellular glycoproteins. Interestingly,  $Le^a$ -containing pollen glycoproteins are detected in each grass species and have high molecular weights, essentially between 75 and 100 kDa. However, because of the structural heterogeneity of  $Le^a$ -containing *N*-glycans, it is difficult to identify species-specific differences in these glycoprotein patterns.

Although our goal here was not to provide a detailed structural analysis of *N*-glycans, in contrast to previously published data (Wilson and Altmann, 1998), our results clearly illustrate that high-mannose-, paucimannosidic- and  $Le^a$ -containing complex-type *N*-glycans are present in grass pollen glycoproteins.

In contrast to protein patterns that show very little species-specific difference owing to the relatively low resolution of SDS–PAGE, patterns obtained for both high-mannose- (Figure 1b) and

complex-type *N*-glycan- (Figure 1c) containing pollen glycoproteins are species-specific. This makes it possible to determine the source of a pollen extract, at least qualitatively, by relying upon the analysis of glycoproteomes, using 1D electrophoresis and commercial probes for glycoprotein detection. In addition, glycoproteins with complex *N*-glycans containing  $\beta$ 1,2 xylose- and  $\alpha$ 1,3 fucose glycoepitopes are good candidates for *in vitro* reactivity with cross-reactive carbohydrate determinant (CCD)-specific IgE, as illustrated later. As a result of the composition of their *N*-glycans, these glycoproteins are susceptible to penetrate in the organism after binding to alveolar macrophages following interaction with C-type lectins and mannose cell surface receptors (Weis *et al.*, 1998).

### Characterization of grass pollen glycoproteomes after 2D electrophoresis

In contrast to protein patterns obtained after SDS–PAGE, species-specific differences are observed between the different grass pollen proteomes after 2D electrophoresis analysis (Figure 2, upper panels). These differences are even more obvious when Western blot replicas of 2D gels are probed either with ConA for the detection of glycoproteins with high-mannose-type *N*-glycans (Figure 2, middle panels) or with antibodies for the immunodetection of glycoproteins with plant complex-type *N*-glycans containing  $\beta$ 1,2 xylose and  $\alpha$ 1,3 fucose residues (Figure 2, lower panels). Subsequent analyses were focused on glycoproteins with complex *N*-glycans. Six independent pollen extracts from each species were resolved in 2D gel electrophoresis, and gels were compared to reach an average glycoproteome map for each studied grass pollen. For each species, the six gels were fused together to form a composite image consisting of spots found (at least) in four gels of six gels by using the ImageMaster 2-DE Elite analysis software (GE Healthcare, Orsay, France) (Figure 3, left panels). The highest number of glycoprotein spots was obtained for *L. perenne* with 66 spots. Patterns containing 44, 40, 30 and 33 glycoprotein spots were observed for *A. odoratum*, *D. glomerata*, *P. pratense* and *P. pratensis*, respectively. These glycoproteomes were compared altogether to identify specific glycoprotein markers in 2D gels for each species. Results are presented in Figure 3 (right panels) and show, as for SDS–PAGE analysis, that grass pollen extracts can be differentiated by their glycoproteomes. Spots corresponding to glycoproteins specific for each grass species are marked with white numbers and range from one (*P. pratensis*) to eight (*D. glomerata*) between species.

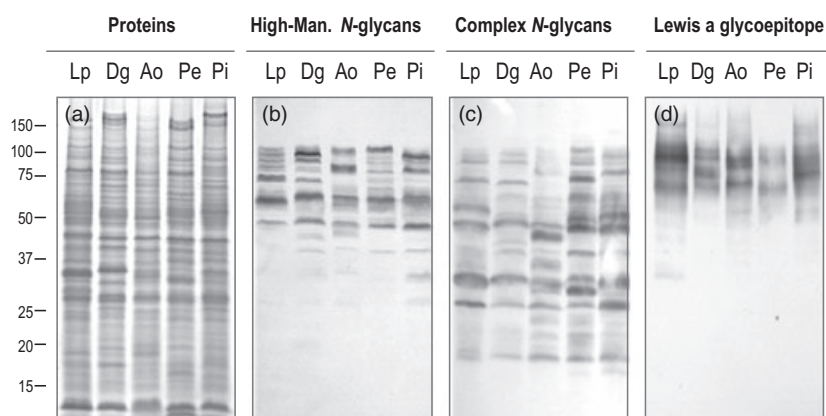


**Figure 1** Grass pollens show different glycoprotein patterns after SDS–PAGE. Thirty microgram of *Lolium perenne* (Lp), *Dactylis glomerata* (Dg), *Anthoxanthum odoratum* (Ao), *Phleum pratense* (Pe) and *Poa pratensis* (Pi) pollen proteins was separated by SDS–PAGE and visualized by Coomassie blue staining (a), or after transfer onto nitrocellulose membrane and concanavalin A affindetection of glycoproteins with high-mannose *N*-glycans (b), or immunodetection of glycoproteins containing complex-type *N*-glycans with  $\beta$ 1,2 xylose and  $\alpha$ 1,3 fucose (c), or Lewis a glycoepitopes (d).

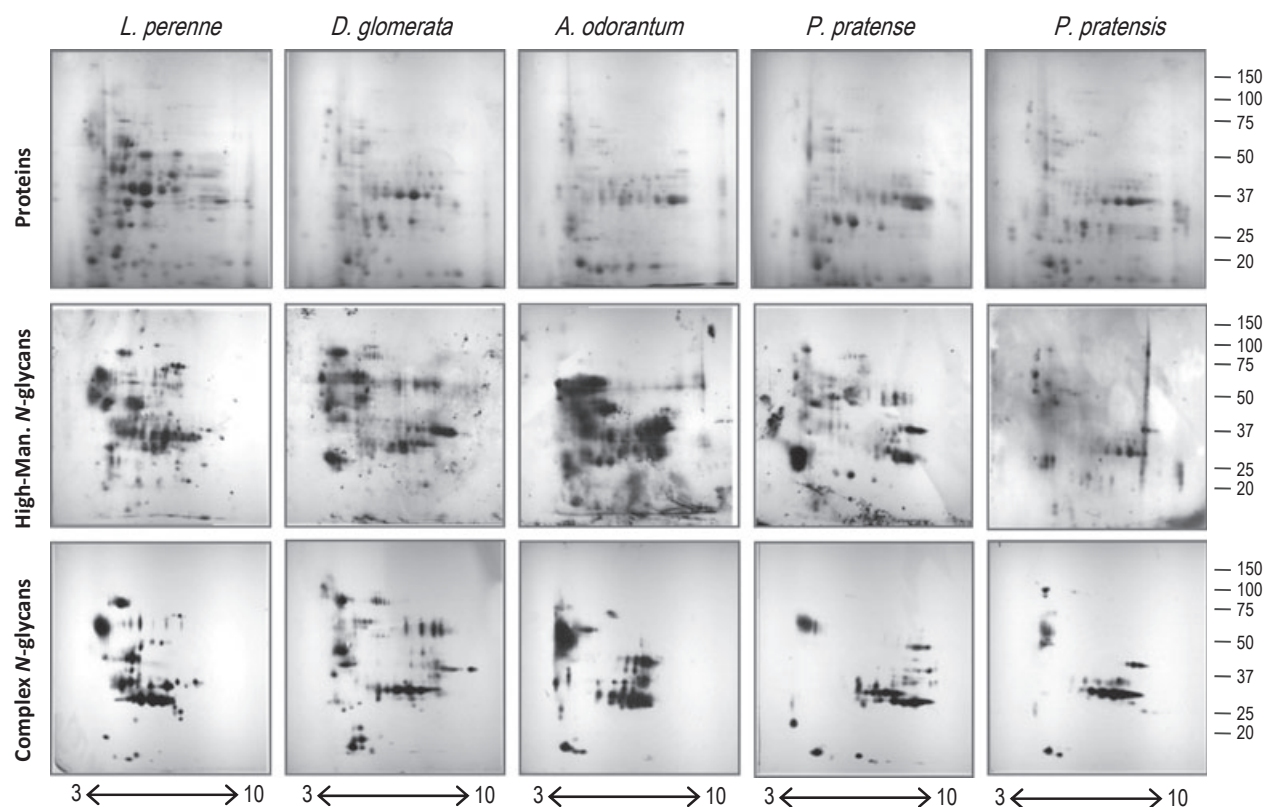


**Figure 2** Grass pollen glycoproteome after 2D electrophoresis. Proteins were separated first in a 3–10 pH gradient, then in 12% polyacrylamide gels and visualized by colloidal blue staining (proteins), or after transfer onto nitrocellulose membrane and concanavalin A immunodetection of glycoproteins with high-mannose *N*-glycans (High-Man. *N*-glycans) or immunodetection of glycoproteins with complex-type *N*-glycans with  $\beta$ 1,2 xylose and  $\alpha$ 1,3 fucose (Complex *N*-glycans).

We subsequently attempted to identify these grass pollen glyco-markers. To this aim, 2D blots detected with anti-plant complex *N*-glycan immunoserum were compared to equivalent 2D gels stained for proteins with colloidal blue. Spots corresponding to species-specific glyco-markers (white numbers in Figure 3, right panels), as well as some spots corresponding to glycoproteins common to different grass pollens (black numbers in Figure 3, right panels), were collected and processed for an identification by mass spectrometry. Results are presented in Table 1. Not all the glyco-markers could be identified. Moreover, despite an approach focused on glycoprotein detection, we mostly identified unglycosylated proteins, likely due to the fact that spots corresponding to glycoproteins overlap with spots corresponding to abundant non-glycosylated protein species, which then overcome signals corresponding to less abundant glycosylated proteins during peptide sequencing by LC-MS/MS. Most of these identified unglycosylated proteins are involved in cellular energetic metabolism and in biosynthesis, assembly or degradation of proteins. We also identified proteins implicated in the cytoskeleton dynamics, in signal transduction and in the cell cycle regulation. The presence of these proteins in pollen is consistent with the development of the pollen tube requesting a fast mobilization of proteins and energy. Using a similar approach, Holmes-Davis *et al.* (2005) have identified 135 proteins in pollen from *Arabidopsis thaliana*, of which 20% are involved in metabolism, 17% in energetic generation and 12% in cellular structure.

Interestingly, we were able to identify some potentially glycosylated proteins within the pollen extracts, i.e. proteins presenting a signal peptide and, at least, a consensus *N*-glycosylation site Asn-X-Ser/Thr, where X can be any amino acid except a Pro. Apart a protein disulfide isomerase in *P. pratensis*, we identified pollen allergens already reported in *L. perenne*, *P. pratense*, or homologs to allergens described in other grass species for *D. glomerata* and *A. odoratum*. They all belong to group 1 or group 11 allergens.

### Most grass pollen allergens are glycoproteins with complex *N*-glycans

To identify the implication of pollen glycoproteins in allergy, we have compared the reactivity of grass pollen proteins with either seric IgE from patients allergic to grass pollen or rabbit antibodies specific for plant complex *N*-glycans. IgE reactivity profiles of 24 sera from grass pollen allergic patients were analysed using protein extracts from pollens of *A. odoratum*, *D. glomerata*, *L. perenn*, *P. pratense* and *P. pratensis*. A typical pattern for IgE reactivity with grass pollen allergens is presented in Figure 4a. Similar patterns were observed with 18 of 24 sera analysed. IgE from these sera react with two main groups of allergens (Figure 4a). A first group of allergens, with molecular masses ranging between 25 and 37 kDa, corresponds to allergens from groups 1 and 5, while a second group, with molecular masses ranging from 50 to 60 kDa, corresponds to group 4 allergens. Except for group 11 allergens from *D. glomerata*, allergens with molecular masses lower than the 18 kDa are not

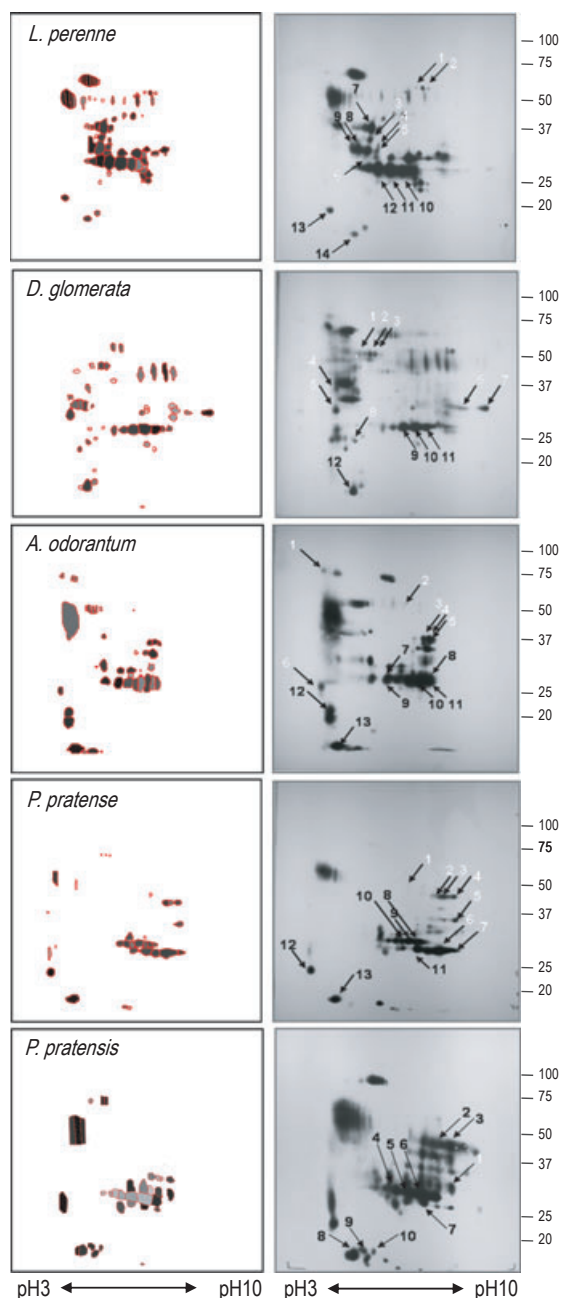


**Figure 3** Major glycoproteins and species-specific glycoprotein markers are identified in the pollen extracts after 2D electrophoresis. Proteins were separated first in a 3–10 pH gradient and then in 12% polyacrylamide gels. After protein transfer onto a nitrocellulose membrane, glycoproteins were immunodetected with antibodies specific for  $\alpha$ 1,3 fucose- and  $\beta$ 1,2 xylose-containing plant complex *N*-glycans. Five grass pollen glycoproteome are presented here: *Lolium perenne*, *Dactylis glomerata*, *Anthoxanthum odoratum*, *Phleum pratense* and *Poa pratensis*. Panels on the right present the complex *N*-glycoproteome for each grass protein. Average maps of glycoproteins were performed by comparison of six independent 2D immunoprints, as those presented in the right panels, using the image master 2DE Elite analysis software. These composite image (left panels) present spots found in more than 60% of gels. Species-specific spots are the ones present in only one grass species composite image. They are indicated by white numbers in right panels. Spots marked with black numbers correspond to glycoproteins that can be found in at least two pollen extracts.

detected here. In contrast, probably due to the extraction of pollen proteins in the presence of detergent, we identified within the timothy grass pollen a new putative 125-kDa allergen reacting with seric IgE from most allergic patients (Pe in Figure 4a).

To compare IgE reactivity profiles with complex *N*-glycoprotein patterns, protein extracts from each pollen species were separated using SDS-PAGE, and after transfer from the gel onto a nitrocellulose membrane, each lane was cut into two parts. One half lane was treated for IgE reactivity with the sera of the same allergic patient as in Figure 4a, while the second half lane was treated with antibodies specific for plant complex *N*-glycans (Figure 4b). As illustrated in Figure 4b (half lanes G), most grass pollen allergens are glycoproteins reacting with rabbit antibodies specific for plant complex *N*-glycans. However, in the present case representing 75% of the analysed population of allergic patients, the observed IgE reactivity pattern is not due to IgE specific for carbohydrate epitopes on pollen glycoproteins. Indeed, periodate oxidation abolished the reaction of *N*-glycan-specific rabbit antibodies with glycoproteins, but only slightly reduced IgE reactivity with the major glycoallergens (Figure 4c).

In conclusion, this experiment clearly confirms that numerous allergens in grass pollens are glycoproteins with complex *N*-glycans containing  $\beta$ 1,2 xylose and  $\alpha$ 1,3 fucose glycoepitopes. Periodate oxidation has only a very limited effect on IgE reactivity with grass pollen allergens for 75% of the allergic patient sera examined. This is indicative for the presence of very little or no IgE specific for complex plant *N*-glycans in these sera. This also illustrates that when performed 'on blot' with mild conditions, periodate oxidation specifically destroys glycoepitopes and does not affect protein-based IgE-binding capacity.

In contrast, we observed that periodate treatment strongly reduces IgE binding to grass pollen glycoproteins in 25% of the human sera analysed so far. For five of 24 tested sera (Figure 5b), patterns obtained after mild periodate treatment are restricted to the detection of the major allergens, as shown in Figure 5a for sera devoid of anti-plant complex *N*-glycan IgE. This highlights the presence of both glycoepitope- and peptide epitope-specific IgE in these sera. Interestingly, one serum of 24 shows a strong IgE reactivity with pollen proteins almost exclusively because of glycan-specific IgE (Figure 5c).

#### Anti-CCD IgEs react with a wide variety of allergen and non-allergen glycoproteins.

While it was studied for a long time (Mari *et al.*, 1999; Hemmer *et al.*, 2001; van Ree, 2002), the clinical relevance of glycan-specific IgE is still a matter of controversy (Malandain, 2005; Altmann, 2007). To appreciate the extent of IgE cross-reactivity in the patient serum showing a strong decrease in IgE reactivity after periodate oxidation (Figure 5c, serum H), we used two model plant glycoproteins with well-defined complex *N*-glycans, not considered as allergens. The first one is PHA-L, the leucoagglutinin form of the bean lectin phytohaemagglutinin (PHA). Subunits constitutive of PHA-L have two major *N*-glycans. A first one, linked to Asn12, is of high mannose type, while the second, a paucimannose-type *N*-glycan, is attached to Asn60 (Rayon *et al.*, 1996). The second model glycoprotein, a 15.5-kDa recombinant chicken avidin produced in maize, is a mixture of glycoforms. Indeed, this recombinant avidin exhibits on its unique *N*-glycosylation site a wide variety of *N*-glycans from simple paucimannose-type *N*-glycan, similar to the ones found on PHA-L, to the most complex Le<sup>a</sup>-containing *N*-glycan (Bardor *et al.*, 2003).

**Table 1** Proteins identified by LC-MS/MS analysis

| Species                     | Spot | Protein name<br>(organism)                                          | Access no.   | Matched peptides<br>(% coverage) | pI/MW<br>(experimental) (kDa) | pI/MW<br>(theoretical) (kDa) |
|-----------------------------|------|---------------------------------------------------------------------|--------------|----------------------------------|-------------------------------|------------------------------|
| <i>Lolium perenne</i>       | 1    | NI                                                                  |              |                                  | 4.6/75                        |                              |
|                             | 2    | NI                                                                  |              |                                  | 4.83/75                       |                              |
|                             | 3    | NI                                                                  |              |                                  | 5.52/44                       |                              |
|                             | 4    | Malate deshydrogenase ( <i>Zea mays</i> )                           | gi 195628708 | 8 (25)                           | 5.64/42                       | 5.77/35.9                    |
|                             | 5    | Phosphoglycerate kinase ( <i>Triticum aestivum</i> )                | gi 3293043   | 20 (50)                          | 5.49/39                       | 5.64/42                      |
|                             | 6    | Succinyl CoA synthetase subunit A ( <i>Oryza sativa</i> )           | gi 75294330  | 14 (35)                          | 5.62/39                       | 5.98/45.4                    |
|                             | 7    | UDP-glucose pyrophosphorylase ( <i>O. sativa</i> )                  | gi 7417426   | 29 (44)                          | 5.55/49                       | 5.46/51.9                    |
|                             | 8    | Succinyl CoA ligase $\beta$ chain ( <i>O. sativa</i> )              | gi 75261432  | 15 (33)                          | 5.17/39.5                     | 5.98/45.4                    |
|                             | 9    | Succinyl CoA ligase $\beta$ chain ( <i>O. sativa</i> )              | gi 75261432  | 15 (33)                          | 5.23/41                       | 5.98/45.4                    |
|                             | 10   | Glyceraldehyde-3-phosphate dehydrogenase ( <i>Hordeum vulgare</i> ) | gi 18978     | 18 (55)                          | 6.2/38                        | 6.20/33.4                    |
|                             | 11   | Glyceraldehyde-3-phosphate dehydrogenase ( <i>H. vulgare</i> )      | gi 18978     | 19 (51)                          | 6.2/31                        | 6.20/33.4                    |
|                             | 12   | Glyceraldehyde-3-phosphate dehydrogenase ( <i>H. vulgare</i> )      | gi 18978     | 5 (26)                           | 6.2/29                        | 6.20/33.4                    |
|                             | 13   | Pollen allergen Lol p 1 precursor ( <i>L. perenne</i> )             | gi 126385    | 21 (54)                          | 5.93/30.6                     | 5.52/28.8                    |
|                             | 14   | Pollen allergen Lol p 1 precursor ( <i>L. perenne</i> )             | gi 126385    | 21 (54)                          | 5.72/31.4                     | 5.52/28.8                    |
|                             | 15   | Pollen allergen Lol p 1 precursor ( <i>L. perenne</i> )             | gi 126385    | 21 (54)                          | 5.66/31.9                     | 5.52/28.8                    |
|                             | 16   | Major pollen Lol p 11 ( <i>L. perenne</i> )                         | gi 47605808  | 11 (44)                          | 5.24/16                       | 5.1/15.2                     |
|                             | 17   | Probable HSP70 ( <i>O. sativa</i> )                                 | gi 21104622  | 23 (28)                          | 4.6/75                        | 5.25/69.3                    |
|                             | 18   | Acid invertase ( <i>L. perenne</i> )                                | gi 124270304 | 17 (34)                          | 4.72/51                       | 4.87/59.6                    |
| <i>Dactylis glomerata</i>   | 1    | Vacuolar ATP synthase subunit A ( <i>Z. mays</i> )                  | gi 195658441 | 12 (22)                          | 5.23/63                       | 5.98/62.2                    |
|                             | 2    | Vacuolar ATP synthase subunit A ( <i>H. vulgare</i> )               | gi 2493123   | 8 (12)                           | 5.41/63                       | 5.98/62.2                    |
|                             | 3    | NI                                                                  |              |                                  | 5.48/63                       |                              |
|                             | 4    | NI                                                                  |              |                                  | 4.48/46                       |                              |
|                             | 5    | Actin 1 ( <i>O. sativa</i> )                                        | gi 15144317  | 16 (43)                          | 4.5/38                        | 5.3/42                       |
|                             | 6    | NI                                                                  |              |                                  | 8/39                          |                              |
|                             | 7    | NI                                                                  |              |                                  | 9.8/39                        |                              |
|                             | 8    | NI                                                                  |              |                                  | 5.14/29                       |                              |
|                             | 9    | Pollen allergen Pha a 1 ( <i>Phalaris aquatica</i> )                | gi 2498576   | 13 (37)                          | 6.13/33                       | 5.5/29.4                     |
|                             | 10   | Pollen allergen Pha a 1 ( <i>P. aquatica</i> )                      | gi 2498576   | 12 (37)                          | 6.45/33                       | 5.5/29.4                     |
|                             | 11   | Pollen allergen Phl p 1 ( <i>Phleum pratense</i> )                  | gi 473360    | 15 (40)                          | 5.93/33                       | 6.14/28.8                    |
|                             | 12   | Major pollen Lol p 11 ( <i>L. perenne</i> )                         | gi 47605808  | 12 (47)                          | 5.09/19                       | 5.1/15.2                     |
|                             | 13   | Enolase 2 ( <i>Z. mays</i> )                                        | gi 1169528   | 11 (31)                          | 4.92/42                       | 5.7/48.4                     |
|                             | 14   | Putative Enolase ( <i>O. sativa</i> )                               | gi 55297212  | 10 (25)                          | 5.03/41                       | 5.72/51.9                    |
|                             | 15   | Aldolase ( <i>O. sativa</i> )                                       | gi 218157    | 13 (21)                          | 6.1/50                        | 6.56/39.1                    |
| <i>Anthoxanthu odoratum</i> | 1    | NI                                                                  |              |                                  | 4.3/103                       |                              |
|                             | 2    | NI                                                                  |              |                                  | 6.03/68                       |                              |
|                             | 3    | UDP-glucose pyrophosphorylase ( <i>O. sativa</i> )                  | gi 7417426   | 12 (25)                          | 6.26/47                       | 5.46/54.9                    |
|                             | 4    | UDP-glucose pyrophosphorylase ( <i>O. sativa</i> )                  | gi 7417426   | 4 (12)                           | 6.36/47                       | 5.46/54.9                    |
|                             | 5    | NI                                                                  |              |                                  | 6.43/46                       |                              |
|                             | 6    | NI                                                                  |              |                                  | 4.14/29                       |                              |
|                             | 7    | Putative cell division cycle protein 48 ( <i>O. sativa</i> )        | gi 110289141 | 9 (14)                           | 5.72/94                       | 5.12/90.4                    |
|                             | 8    | Enolase 2 ( <i>Z. mays</i> )                                        | gi 1169528   | 14 (39)                          | 5.36/69                       | 5.7/48.4                     |
|                             | 9    | Enolase ( <i>Z. mays</i> )                                          | gi 226499996 | 15 (40)                          | 5.42/68                       | 5.32/48.3                    |
|                             | 10   | UDP-glucose pyrophosphorylase ( <i>O. sativa</i> )                  | gi 7417426   | 25 (39)                          | 5.57/50                       | 5.46/51.9                    |
|                             | 11   | 3-phosphoglycerate kinase ( <i>Triticum urartu</i> )                | gi 28172905  | 6 (25)                           | 5.57/36                       | 4.98/31.4                    |
|                             | 12   | Reversibly glycosylated polypeptide ( <i>T. aestivum</i> )          | gi 4158232   | 10 (32)                          | 5.83/34                       | 5.82/42                      |
|                             | 13   | Aldolase ( <i>O. sativa</i> )                                       | gi 218157    | 8 (19)                           | 5.98/35                       | 6.56/39.1                    |
|                             | 14   | Aldolase ( <i>O. sativa</i> )                                       | gi 218157    | 6 (21)                           | 6.29/35                       | 6.56/39.1                    |
|                             | 15   | Pollen allergen Lol p 1 ( <i>L. perenne</i> )                       | gi 168314    | 4 (20)                           | 5.98/33                       | 5.38/27.7                    |
|                             | 16   | Triose-phosphate isomerase ( <i>H. vulgare</i> )                    | gi 2507469   | 7 (25)                           | 6.32/40                       | 5.39/26.9                    |
|                             | 17   | Pollen allergen Pha a 1 precursor ( <i>P. aquatica</i> )            | gi 2498576   | 12 (34)                          | 6.4/31                        | 5.5/29.4                     |
|                             | 18   | Pollen allergen Pha a 1 precursor ( <i>P. aquatica</i> )            | gi 2498576   | 12 (34)                          | 6.4/30                        | 5.5/29.4                     |
|                             | 19   | Pollen allergen Pha a 1 precursor ( <i>P. aquatica</i> )            | gi 2498576   | 11 (39)                          | 6.23/32                       | 5.5/29.4                     |
|                             | 20   | Group 1 allergen Dac g 1.01 precursor ( <i>D. glomerata</i> )       | gi 33149333  | 8 (28)                           | 5.94/31                       | 5.91/26.5                    |
|                             | 21   | 14-3-3 protein homolog ( <i>H. vulgare</i> )                        | gi 100554    | 5 (20)                           | 4.80/25                       | 4.83/29.4                    |

Table 1 Continued

| Species              | Spot | Protein name<br>(organism)                                        | Access no.   | Matched peptides<br>(% coverage) | pI/MW<br>(experimental) (kDa) | pI/MW<br>(theoretical) (kDa) |
|----------------------|------|-------------------------------------------------------------------|--------------|----------------------------------|-------------------------------|------------------------------|
| <i>P. pratense</i>   | 22   | Pollen allergen Lol p 11 ( <i>L. perenne</i> )                    | gi 47605808  | 9 (38)                           | 4.94/18                       | 5.1/15.2                     |
|                      | 1    | Enolase ( <i>O. sativa</i> )                                      | gi 55297212  | 5 (17)                           | 5.26/57                       | 5.32/48.3                    |
|                      | 2    | NI                                                                |              |                                  | 7.66/48                       |                              |
|                      | 3    | NI                                                                |              |                                  | 8.01/48                       |                              |
|                      | 4    | NI                                                                |              |                                  | 8.5/48                        |                              |
|                      | 5    | NI                                                                |              |                                  | 8.6/39                        |                              |
|                      | 6    | NI                                                                |              |                                  | 7.91/30                       |                              |
|                      | 7    | NI                                                                |              |                                  | 8.62/30                       |                              |
|                      | 8    | Aldolase ( <i>O. sativa</i> )                                     | gi 218157    | 8 (18)                           | 7.93/39                       | 6.56/39.1                    |
|                      | 9    | Glyceraldehyde-3-phosphate<br>dehydrogenase ( <i>H. vulgare</i> ) | gi 18978     | 10 (37)                          | 7.16/39                       | 6.2/33.4                     |
|                      | 10   | Glyceraldehyde-3-phosphate<br>dehydrogenase ( <i>H. vulgare</i> ) | gi 18978     | 16 (42)                          | 7.6/35                        | 6.2/33.4                     |
|                      | 11   | Pollen allergen Phl p 1 precursor<br>( <i>P. pratense</i> )       | gi 1171008   | 12 (41)                          | 6.63/32                       | 6.14/28.8                    |
|                      | 12   | Pollen allergen Phl p 1 ( <i>P. pratense</i> )                    | gi 3901094   | 13 (41)                          | 6.61/30                       | 7.53/28.6                    |
|                      | 13   | Pollen allergen Phl p 1 ( <i>P. pratense</i> )                    | gi 3901094   | 7 (48)                           | 5.2/17                        | 4.96/16.1                    |
| <i>Poa pratensis</i> | 1    | Aldolase ( <i>O. sativa</i> )                                     | gi 218157    | 13 (28)                          | 7.39/34                       | 6.56/39.1                    |
|                      | 2    | Protein disulphide isomerase<br>precursor ( <i>H. vulgare</i> )   | gi 1709617   | 9 (15)                           | 4.85/68                       | 5.02/56.1                    |
|                      | 3    | UDP-glucose pyrophosphorylase<br>( <i>H. vulgare</i> )            | gi 1212996   | 21 (42)                          | 5.3/50                        | 5.2/51.8                     |
|                      | 4    | Aldolase ( <i>O. sativa</i> )                                     | gi 218157    | 12 (25)                          | 6.46/41                       | 6.56/39.1                    |
|                      | 5    | Aldolase ( <i>O. sativa</i> )                                     | gi 218157    | 13 (27)                          | 6.77/40                       | 6.56/39.1                    |
|                      | 6    | Aldolase ( <i>O. sativa</i> )                                     | gi 218157    | 11 (24)                          | 6.41/35                       | 6.56/39.1                    |
|                      | 7    | Glyceraldehyde-3-phosphate<br>dehydrogenase ( <i>H. vulgare</i> ) | gi 18978     | 18 (55)                          | 6.2/37                        | 6.2/33.4                     |
|                      | 8    | Glyceraldehyde-3-phosphate<br>dehydrogenase ( <i>H. vulgare</i> ) | gi 18978     | 18 (50)                          | 6.83/35                       | 6.2/33.4                     |
|                      | 9    | Malate dehydrogenase ( <i>O. sativa</i> )                         | gi 119395240 | 10 (25)                          | 5.98/37                       | 5.75/35.9                    |
|                      | 10   | Succinyl CoA synthase $\beta$ subunit<br>( <i>O. sativa</i> )     | gi 75294330  | 20 (40)                          | 5.74/38                       | 5.98/45.4                    |
|                      | 11   | Pollen allergen Pha a 1 ( <i>P. aquatica</i> )                    | gi 1246116   | 12 (40)                          | 5.99/33                       | 5.5/29.4                     |
|                      | 12   | Pollen allergen Phl p 1 ( <i>P. pratense</i> )                    | gi 473360    | 17 (51)                          | 6.2/33                        | 6.14/28.8                    |
|                      | 13   | Pollen allergen Phl p 11 ( <i>P. pratense</i> )                   | gi 23452313  | 13 (61)                          | 5.28/19                       | 4.96/16.1                    |
|                      | 14   | Pollen allergen Phl p 11 ( <i>P. pratense</i> )                   | gi 23452313  | 7 (38)                           | 5.41/20                       | 4.96/16.1                    |

NI, not identified. Proteins indicated in green correspond to species-specific spots indicated by arrows and white numbers on 2D gels presented in Figure 3.

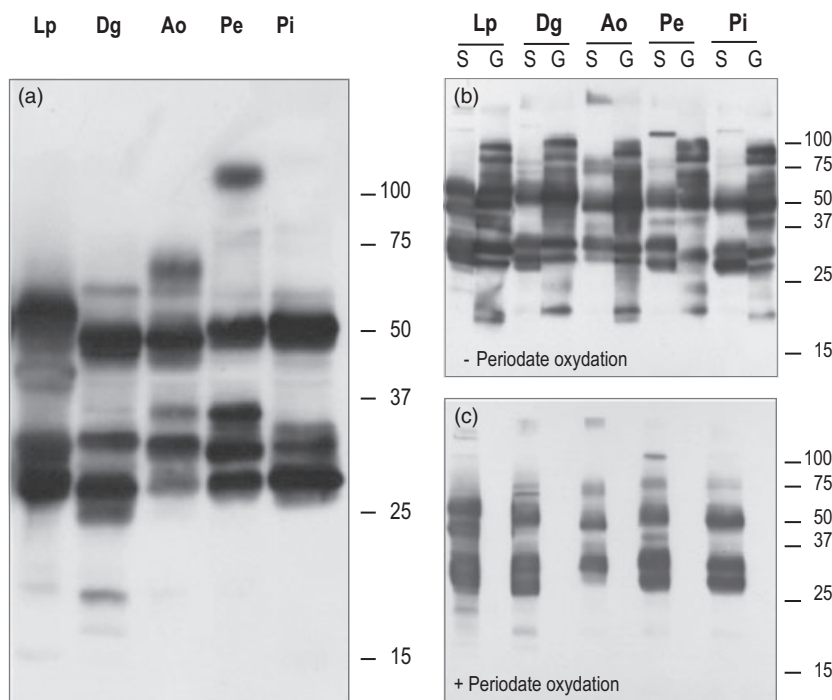
As illustrated in Figure 6, glycan-specific IgE from serum H react strongly with PHA-L and with recombinant chicken avidin produced in maize, two glycoproteins having typical paucimannosidic *N*-glycans. Consistent with the results presented in Figure 5c, the IgE reactivity was completely abolished for PHA-L and strongly reduced for recombinant avidin after periodate oxidation treatment. The inhibition of the IgE binding after glycan disruption confirms their specificity for  $\alpha$ 1,3 fucose- and  $\beta$ 1,2 xylose-containing glycoepitopes in paucimannose-type oligosaccharides *N*-linked to these model glycoproteins. However, in contrast to the presence of many glycoproteins containing Lewis a glycoepitopes in grass pollen extracts (Figure 1d), IgE from sera of grass pollen allergic patients do not cross-react with BSA coupled with the Lewis a epitope (data not shown). This result suggests that the Le<sup>a</sup> structure is not capable to induce an IgE response in allergic patients.

The human sera selected for the illustration of glycan-specific IgE reactivity in Figure 5 shows different levels of cross-reactivity

with two glycoproteins of 20 and 50 kDa detected in venoms of *Apis mellifera* and *Vespula flavopilosa*, respectively. These two glycoproteins are also detected with a rabbit polyclonal serum specific for  $\alpha$ 1,3 fucose-/ $\beta$ 1,2 xylose-containing plant complex *N*-glycans (Figure 7b). They probably correspond to phospholipase A2 from bee venom and hyaluronidase from wasp *V. flavopilosa* venom. These two allergens from hymenoptera venom are glycosylated with *N*-glycans containing a  $\alpha$ 1,3 fucose glycoepitope also found in plant complex *N*-glycans (Bardor *et al.*, 2003; Kolarich *et al.*, 2005), which probably explains a glycan-specific cross-reactivity with pollen glycoallergens. In contrast, using either glycan-specific rabbit antibodies or human IgE, we have been unable to detect glycoproteins in the venom from *Polistes fuscatus*.

Altogether, these results illustrate a false positive *in vitro* diagnosis of insect venom allergy for 25% grass pollen allergic patients, because of the presence of IgE specific for plant complex *N*-glycans in their sera.

**Figure 4** Most grass pollen allergens are glycoproteins with complex *N*-glycans. Proteins were separated by SDS-PAGE and transferred onto nitrocellulose membrane. Allergens were immunostained with allergic patients sera. Seventy-five per cent of the 24 tested sera show the typical reactivity pattern illustrated in panel (a). *N*-glycosylation of these allergens was verified by a co-revelation with the serum of an allergic patient sensitized to grass pollens (S) and a rabbit with immunoserum specific for plant complex *N*-glycans (G) without (b) or with periodate oxidation (c). *Lolium perenne* (Lp), *Dactylis glomerata* (Dg), *Anthoxanthum odoratum* (Ao), *Phleum pratense* (Pe) and *Poa pratensis* (Pi).



## Conclusion

In Europe, grass pollen immunotherapy accounts for one-third of specific immunotherapies against type I allergies. It is usually performed with a mixture of grass pollens rather than with a single pollen (Bousquet *et al.*, 1998, 2001). The most widely prescribed combination is a pollen mixture from *Anthoxanthum odoratum*, *D. glomerata*, *L. perenne*, *P. pratensis* and *P. pratense*, which all belong to the Pooideae subfamily (i.e. common grasses) (Didier *et al.*, 2007; Moingeon *et al.*, 2008). Eleven groups of grass pollen allergens with similar properties present in different species are currently defined (Pauli, 2000; Weber, 2004). Numerous studies evaluating the reactivity of patients' sera with various pollen extracts have demonstrated a strong IgE cross-reactivity between Pooideae species (van Ree *et al.*, 1992, 1998; Andersson and Lidholm, 2003; Mari, 2003). Immunological cross-reactivity between Pooideae pollens results from molecular similarities between allergens, with some of those allergens, such as group 1 and group 5 exhibiting a 90% and 55%–80% homology at the amino acid level between distinct species (Ferreira *et al.*, 2004). Nonetheless, a detailed analysis of those allergens has revealed the existence of species-specific T- or B-cell epitopes associated with those molecules (Schenk *et al.*, 1995; Weber, 2004; Linhart *et al.*, 2005; Chabre *et al.*, 2009).

Herein, we assessed the contribution of carbohydrate recognition by IgE to grass pollen allergenicity. More precisely, glycoprotein analysis using 1D and 2D electrophoresis coupled with glycoprotein detection on Western blots has allowed not only to obtain patterns specific for each grass pollen but also the identification of some species-specific markers and part of the glycoproteome. Interestingly, numerous grass pollen allergens are glycoproteins, which could explain why about 25% of grass pollen allergic patients produce IgE mainly or eventually exclusively specific for plant complex *N*-glycans. Together with

homologies in their amino acid sequences, glycan-specific IgE are also responsible in part for the immunological cross-reactivity observed between grass pollen allergens. Furthermore, these IgE also cross-react with either non-allergen plant glycoproteins or glycoproteins from hymenoptera venom containing the same glycoepitopes.

Our study also provides support for a role of such glycoepitopes in IgE responses observed in some patients. In this regard, the 24 sera analysed in this study can be classified into two different groups. A first group (18 sera) comprises sera containing little or no IgE specific for plant complex *N*-glycans and characterized by little or no modification of the immunodetection pattern after periodate oxidation (Figure 5a). In the second group, six sera show an altered pattern after periodate oxidation (five sera) and contain both peptide- and glycan-specific IgE, with even one serum containing almost exclusively glycan-specific IgE and presenting a strong sensitivity to periodate oxidation (Figure 5c). In this second group, IgE binding to pollen proteins is mainly due to the serum reactivities with cross-reacting carbohydrate determinants (CCDs). Altogether, 25% of pollen allergic patients have developed IgE directed against plant complex *N*-glycans. Our results are consistent with previous data illustrating the reactivity of IgE specific for *N*-glycans in the sera of patients suffering of allergy to celery (Bublin *et al.*, 2003), olive pollen (Batanero *et al.*, 1999) and grass pollens (Fötisch and Vieths, 2001).

Altogether, our results lead to two major conclusions. First, as previously suggested in Chardin *et al.* (2008), the use of glycoepitope-free recombinant allergens is needed to increase the specificity of *in vitro* allergy diagnosis. Second, a mixture of pollens from common and well-characterized Pooideae such as *A. odoratum*, *D. glomerata*, *L. perenne*, *P. pratense* and *P. pratensis* is appropriate for grass pollen-specific immunotherapy, as it ensures a consistent and well-balanced composition in glycoepitopes linked with allergens and reflects natural exposure and sensitization conditions.

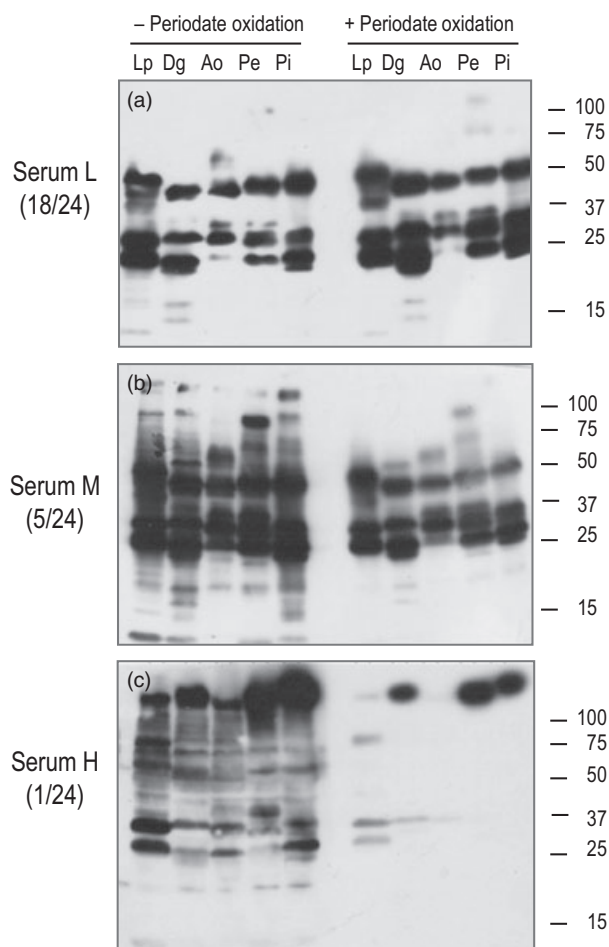


**Figure 5** Twenty-five per cent of allergic patient sera contain IgE specific for carbohydrate determinant(s). Typical patterns of grass allergens observed after immunodetection with sera from patients allergic to grass pollens without and with periodate oxidation. Twenty-four sera have been tested on grass pollens extracts from *Lolium perenne* (Lp), *Dactylis glomerata* (Dg), *Anthoxanthum odoratum* (Ao), *Phleum pratense* (Pe) and *Poa pratensis* (Pi). The pollen allergic patient sera used here are representative of 18/24 sera containing low or undetectable (L), 5/24 medium (M) or 1/24 high (H) amounts of IgE specific for cross-reacting carbohydrate determinant.

## Experimental procedures

### Reagents

Recombinant chicken avidin produced in maize was purchased from Sigma (Saint Quentin Fallavier, France). Phytohaemagglutinin A (PHA) from *Phaseolus vulgaris* was from Vector laboratories, and chicken avidin purified from egg white was from Zymed Laboratories Inc. (distributed by CliniSciences, Montrouge, France). Venoms from *A. mellifera*, *P. fuscatus* and *V. flavopilosa* were provided by Stallergenes SA (Antony, France).

Polyclonal sera and purified antibodies specific for  $\alpha$ 1,3 fucose-,  $\beta$ 1,2 xylose- and Lewis a-containing plant complex *N*-glycans were prepared as described in Faye *et al.* (1993) and Fitchette-Lainé *et al.* (1997), respectively. A commercial anti-horseradish peroxidase (HRP) rabbit serum (ref ABIN293976; Antibodies-online GmbH, Aachen, Germany) was

also used for plant complex *N*-glycan immunodetection as previously described in Fitchette *et al.* (2009). Twenty-four sera of grass pollen-sensitive patients provided by Stallergenes SA were used individually for IgE-binding immunoblotting assays. HRP colour development reagent and HRP-labelled goat anti-rabbit IgG were purchased from Bio-Rad Laboratories (Marnes la Coquette, France). HRP-labelled goat anti-human IgE antibodies were from KPL (Baltimore, Maryland).

### SDS-PAGE and blotting

Grass pollens from *L. perenne*, *D. glomerata*, *A. odoratum*, *P. pratense* and *P. pratensis* were provided by Stallergenes SA in a dry form and stored at  $-20^{\circ}\text{C}$  until use. Protein extracts from grass pollens were prepared for SDS-PAGE by homogenization of 100-mg pollen in 1 mL of denaturing buffer [62.8 mM Tris, pH 6.8, containing 15% (v/v) glycerol, 285 mM  $\beta$ -mercaptoethanol and 1% (w/v) sodium dodecyl sulphate (SDS)]. Homogenates were boiled for 10 min and then centrifuged at  $10\,000 \times g$  for 10 min. Supernatants were collected and stored at  $-20^{\circ}\text{C}$  until SDS-PAGE analysis.

SDS-PAGE was performed according to the method of Laemmli (1970) in 15%T, 0.5%C acrylamide gels. After electrophoresis, proteins were either visualized by Coomassie blue staining or electrophoretically transferred onto nitrocellulose membranes for immuno- or affino-detection, in 25 mM Tris, 192 mM glycine and 10% (v/v) ethanol, for 2 h at 22 V.

After protein transfer, membranes used for the detection of glycoproteins containing high-mannose-type *N*-glycans were blocked with Tris-buffered saline (TBS: 20 mM Tris, pH 7.4, containing 0.5 M NaCl) containing 0.1% Tween 20 (TTBS) for 1 h. Then, membranes were incubated at room temperature for 1 h with Con A (25  $\mu\text{g}/\text{mL}$ ) prepared in TTBS containing 1 mM  $\text{CaCl}_2$  and 1 mM  $\text{MgCl}_2$ . After washing with TTBS, membranes were incubated again at room temperature for 1 h with horseradish peroxidase (50  $\mu\text{g}/\text{mL}$ ) in TTBS containing, 1 mM  $\text{CaCl}_2$  and 1 mM  $\text{MgCl}_2$ . After washing, the peroxidase reaction was developed with the HRP colour development reagent (30 mg dissolved in 10 mL methanol) mixed with 50 mL TBS containing 0.06% (v/v) hydrogen peroxide.

For the detection of glycoproteins containing complex *N*-glycans, membranes were blocked with 3% (w/v) skim milk in TBS for 1 h. Subsequently, membranes were incubated at room temperature for 1 h with either a commercial rabbit polyclonal antisera specific for plant complex *N*-glycans (ref ABIN293976) (1 : 3000) or with purified antibodies specific for  $\alpha$ 1,3 fucose-,  $\beta$ 1,2 xylose- and Lewis a-containing plant complex *N*-glycans, diluted 1 : 1000. After washing with TTBS, HRP-labelled goat anti-rabbit IgG (Bio-Rad Laboratories) diluted 1 : 4000 was used as secondary antibody for 1-h incubation. The peroxidase reaction was developed using the HRP colour development reagent, as described earlier.

For IgE immunostaining assays, after blocking with 1% (w/v) skim milk in TBS for 1 h at room temperature, membranes were washed with TTBS and incubated overnight at room temperature with individual sera from grass pollen allergic patients diluted 1 : 20 in TTBS containing 0.2% skim milk. After washing, HRP-labelled goat anti-human IgE antibodies were used as secondary antibodies (diluted 1 : 2500). The peroxidase reaction was developed with the ECL Western blotting reagent (GE Healthcare) using Kodak X-Omat AR Film sheets (Sigma).

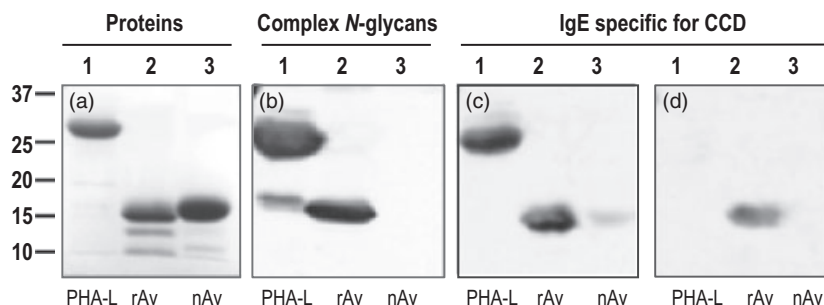


**Figure 6** Anti-glycan IgE cross-react with plant natural or recombinant glycoproteins containing complex *N*-glycans. The model glycoproteins [PHA-L, plant-made chicken avidin (rAv) and natural chicken avidin (nAv)] were separated by SDS-PAGE and visualized by Coomassie blue staining (a), or after transfer onto a nitrocellulose membrane and immunodetection either with a rabbit immunoserum specific for plant complex-type *N*-glycans containing  $\beta$ 1,2 xylose and/or  $\alpha$ 1,3 fucose (b), or with the serum of a pollen allergic patients containing IgE specific for cross-reacting carbohydrate determinant (serum H presented in Figure 5c). Panel (d) was treated for immunodetection as for c but after periodate oxidation.

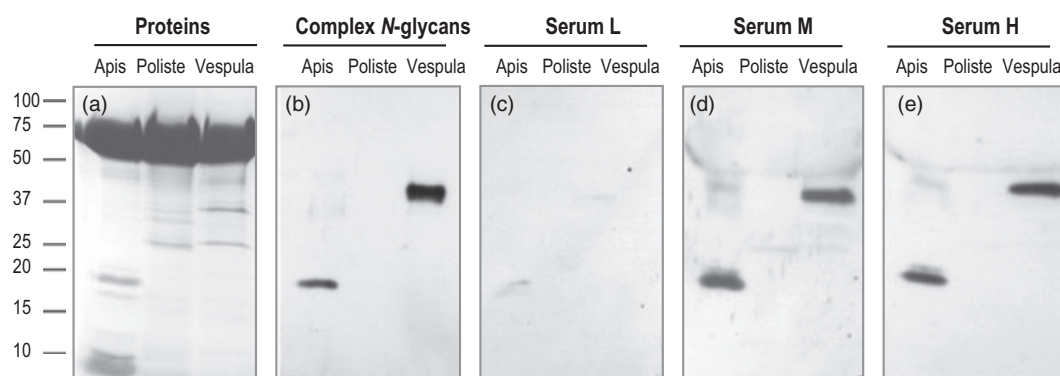


**Figure 7** Cross-reactivity of IgE from grass allergic patients sera with glycoallergens from venoms of *Apis mellifera*, *Polistes fuscatus* and *Vespula flavopilosa*. Proteins from hymenoptera venoms were separated by SDS-PAGE and visualized by Coomassie blue staining (a), or after transfer onto a nitrocellulose membrane and immunodetection with either a rabbit immunoserum specific for plant complex-type *N*-glycans (b) or with sera from pollen allergic patients containing high (H), medium (M) or low to undetectable (L) cross-reacting carbohydrate determinants-specific IgE. The human sera used for this experiment are the same as for Figure 5.

### Periodate oxidation

Periodate oxidation was performed 'on blot' after protein transfer and membrane blocking, as in Fitchette *et al.* (2009). After equilibration in 0.1 M acetate buffer (pH 4.5), membranes were incubated for  $2 \times 30$  min in 100 mM sodium metaperiodate diluted in the same acetate buffer, in the dark at room temperature. After washing with acetate buffer, membranes were equilibrated in phosphate buffer saline pH 7.4 (PBS) and placed in a solution of 50 mM sodium borohydride in PBS for 30 min at room temperature. After rinsing with PBS and TBS successively, membranes were immunostained as described previously.

### Two-dimensional gel electrophoresis and blotting

Grass pollen protein extracts were prepared by homogenization of pollens in 2% DTT (prepared in water). These homogenates were incubated 30 min on ice with occasional vortexing. Then, homogenates were centrifuged for 30 min at  $16\,000 \times g$ . Supernatants (S1) were collected, and pellets were treated for a second extraction in the same conditions to get S2. Proteins were precipitated from S1 + S2 in the presence of 12.5% TCA at 4 °C during 1 h. After centrifugation (15 min at  $12\,000 \times g$ ), protein pellets were washed with ice cold acetone and solubilized in a solution containing 9 M urea, 2% (w : v) 3-((3-cholamidopropyl)-dimethylammonio)-1-propane sulfonate (CHAPS) and

1% (w/v) dithioerythritol (DTE). After a suitable dilution, the protein content was determined according to the method of Bradford (1976) with bovine serum albumin as a standard.

One hundred micrograms of grass pollen proteins was added to an isoelectric focusing (IEF) solution, containing 9 M urea, 2% (w/v) CHAPS, 1% (w/v) DTE, 1% (v/v) carrier ampholytes pH 3–10 NL (GE HealthcareLife Science, Orsay, France) and 0.4% w/v bromophenol blue and applied to a 2D electrophoretic run. The first-dimension gel separation was carried out on a nonlinear wide-range immobilized pH named gradient gel Immobiline Dry Strips NL (13 cm, pH 3–10; GE Healthcare) with a horizontal electrophoresis apparatus (Multiphor II; GE Healthcare). After separation, proteins contained in the gel strips were reduced by a 15-min incubation in 10 mM Tris, pH 6.8, containing 6 M urea, 1% SDS, 30% glycerol and 33 mM DTT and alkylated in 245 mM iodoacetamide for 15 more min. The second dimension separation was a SDS-PAGE in a 12.0%T, 0.4%C polyacrylamide resolving gel using a Protean Ixi Cell (Bio-Rad). After migration, proteins were electrophoretically transferred onto nitrocellulose membranes, for immuno- or affinodetection, with a semi-dry system (Nova Blot; GE Healthcare), in 24.2 mM Tris, 77.4 mM glycine, 1.3 mM SDS and 20% (v/v) methanol, for 2 h at 0.8 mA cm<sup>2</sup>. Immuno- or affinodetections were performed as previously described with chemiluminescent revelation.



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