

Characteristics and Immunobiology of Grass Pollen Allergens

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Key Words

Grass pollen · Allergen · Recombinant allergen

Abstract

Grass pollens are one of the most important airborne allergen sources worldwide. About 20 species from five subfamilies are considered to be the most frequent causes of grass pollen allergy, and the allergenic relationships among them closely follow their phylogenetic relationships. The allergic immune response to pollen of several grass species has been studied extensively over more than three decades. Eleven groups of allergens have been identified and described, in most cases from more than one species. The allergens range from 6 to 60 kD in apparent molecular weight and display a variety of physicochemical properties and structures. The most complete set of allergens has so far been isolated and cloned from *Phleum pratense* (timothy grass) pollen. Based on the prevalence of IgE antibody recognition among grass pollen-sensitized individuals, several allergens qualify as major, but members of two groups, groups 1 and 5, have been shown to dominate the immune response to grass pollen extract. Isoform variation has been detected in members of several of the allergen groups, which in some cases can be linked to observed genetic differences. N-linked glycosylation occurs in members of at least three groups. Carbohydrate-

reactive IgE antibodies have been attributed to grass pollen sensitization and found to cross-react with glycan structures from other allergen sources, particularly vegetable foods. Another cause of extensive cross-reactivity are the group 12 allergens (profilins), which belong to a family of proteins highly conserved throughout the plant kingdom and present in all tissues. Members of eight allergen groups have been cloned and expressed as recombinant proteins capable of specific IgE binding. This development now allows diagnostic dissection of the immune response to grass pollen with potential benefits for specific immunotherapy.

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Introduction

While grasses belong to the ecologically most successful and economically most important group of plants, they are also a common cause of allergic disease worldwide. The Poaceae family comprises about 9,000 species, and its members occur naturally in virtually every habitat throughout the world. They are cultivated for food (e.g. cereals, corn, rice, sugar cane) and animal forage, kept as managed surface vegetation on recreational areas and used as turf to prevent soil erosion. Contributing to the competitive advantage and versatility of grasses is the location of the leaf meristem at the base of the blade, an

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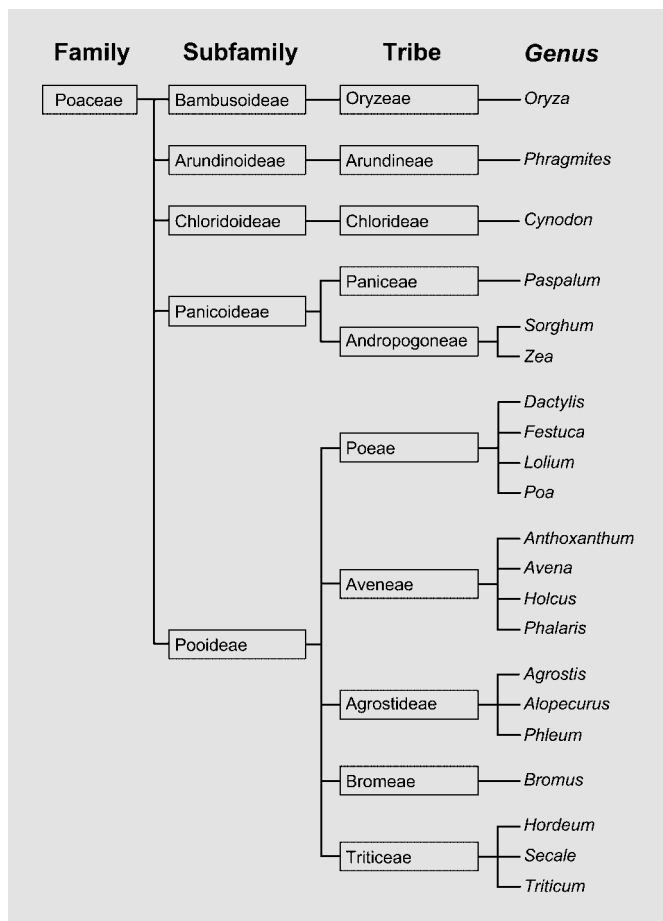


Fig. 1. Phylogenetic relationships (as in Lewis et al. [1]) between grass genera representing important allergenic species.

arrangement which allows continued growth after grazing or other forms of damage to the leaf, such as fire or drought [1, 2].

Most grass species are wind pollinated and release pollen grains in great abundance during a short period of time [1–3]. Heavy loads of airborne grass pollen and intense airway exposure therefore occur during peak flowering season, commonly around early to mid-summer, and allergic sensitization to grass pollen may affect as many as 20% of the general population and up to 40% of atopic individuals. Symptoms of grass pollen allergy are most often distinctly seasonal and predominantly consist of rhinitis and/or conjunctivitis [4–6].

Grass pollen allergens have been studied extensively since the 1960s, and allergenic pollen proteins have been isolated and described from a number of grass species. This paper will provide a brief account of the allergenic

relationships between major representatives of grass species and a review of molecular aspects of grass pollen allergens described to date. It will also describe some characteristics of the IgE antibody response in grass pollen sensitization.

Allergenic Diversity among Grass Species

A limited number of grass species are recognized as major causes of allergic disease, and in clinical practice worldwide, pollen extracts of 20–30 grasses are frequently used for routine diagnosis of grass pollen allergy. The majority of these are species of the temperate region belonging to the Pooideae subfamily. Other genera, representing important allergenic species of the temperate and subtropical zones, belong to the subfamilies Panicoideae, Chloridoideae, Arundinoideae and Bambusoideae. Figure 1 shows the taxonomic classification of 20 common allergenic grass genera according to Lewis et al. [1].

How diverse or similar are the common grass species with respect to their pollen allergen content and the allergic sensitization they cause? The extent of allergenic similarity between 17 species represented in figure 1 was assessed by comparing specific IgE antibody determinations in sera of 143 grass pollen-sensitized individuals. Measurements were made with ImmunoCAP™ tests for the different grasses, and the data set was analyzed by first plotting the values for each species against those for *Phleum pratense* (fig. 2a). While this analysis may not reveal differences in individual epitopes, it appeared that the IgE determinations for *P. pratense* were very similar to those for 12 of the other compared species. The data for the four species *Cynodon dactylon*, *Phragmites communis*, *Sorghum halepense* and *Paspalum notatum*, which stood out as different from *P. pratense*, were then plotted against each other, whereby two (*S. halepense* and *P. notatum*) were found to be similar (fig. 2b–d).

This simple survey suggests that among the 17 grass species compared, four groups exist within which the species produce pollen with similar overall allergen content. Although the results may have been influenced by the geographical representation of the serum donors in this case, there is nevertheless a striking correlation with the botanical relationships among the species included. In agreement with the proposition by van Ree et al. [7], the data indicate that a minimal set of pollen extracts necessary for diagnosis of grass pollen allergy could be made considerably smaller than the number currently being used.

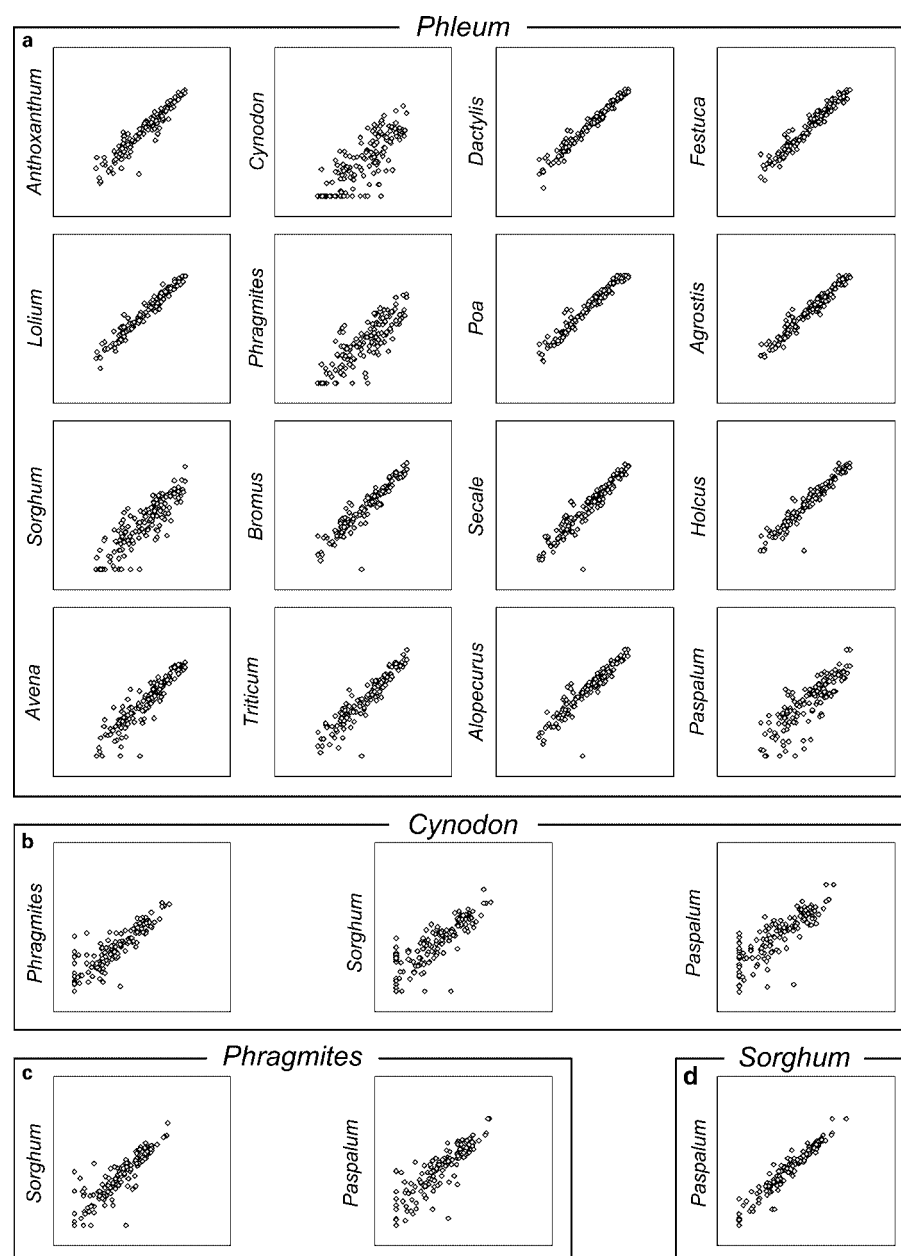


Fig. 2. Comparison of specific IgE to pollen extracts of 17 grass species in sera of 143 grass pollen-sensitized individuals. a Concentrations of specific IgE to each given grass pollen extract were plotted against those to *P. pratense*. Species which appeared to be significantly different from *P. pratense* were then plotted against each other in order to detect allergenic similarity. b–d x-axis values are for *C. dactylon* (b), *P. communis* (c) and *S. halepense* (d).

Grass Pollen Allergens

The bulk of allergenicity of grass pollen can be attributed to a limited number of proteins which are rapidly released from the pollen grain upon hydration [8–11]. To date, eleven different groups of grass pollen allergens have been identified and characterized from one or more species [12] (table 1). These groups represent a variety of glycosylated and non-glycosylated proteins of different sizes, structures and physicochemical properties.

Many grass pollen allergens have been shown to exist as multiple isoforms, distinguishable by size- or charge-dependent separation techniques or by their primary structure. Such isoform variation may arise in different ways, through the existence of multiple gene products, by differential modification of a single gene product or by a combination of multiple gene products and modification(s). Modifications that may result in electrophoretic heterogeneity include partial removal of the polypeptide chain (processing, cleavage, degradation) and glycosyla-

Table 1. Grass pollen allergens cloned and/or characterized to date

Species	Common name	Sub-family	Allergen designation	MW, kD (exp.)	MW, kD (calc.)	pI (exp.)	pI (calc.)	CHO	Function or similarity	Cloned	Acc. No.	Reference
<i>Oryza sativa</i>	rice	Bam	Ory s 1	35	26.3	n.d.	8.58	yes ¹	β-expansin	yes	AAA86533	15
			Ory s 12	n.d.	14.2	n.d.	4.91	no	profilin	yes	AAG32056	158
<i>Cynodon dactylon</i>	Bermuda grass	Chl	Cyn d 1	31–34	26.8	5.6–7.4, 10	8.56	yes	β-expansin	yes	O04701	22, 42, 187
			Cyn d 2	n.d.	11.2	n.d.	4.74	no		yes	CAA10346	188
			BG-60	60 ²	–	9.7	–	28%	flavoprotein	no	–	87, 88
			Cyn d 7	12	9	n.d.	4.24	no	Ca ²⁺ -binding	yes	P94092	127, 128
			Cyn d 12	14	14.1	n.d.	4.89	no	profilin	yes	O04725	157
<i>Sorghum halepense</i>	Johnson grass	Pan	Sor h 1	35	28.1	n.d.	7.5	yes ¹	β-expansin	yes	–	38
<i>Zea mays</i>	maize	Pan	Zea m 1	28–31	26.9	n.d.	9.04	yes ¹	β-expansin	yes	P58738	39
			Zmc13	n.d.	15.7	n.d.	4.52	yes ¹	soybean trypsin inhibitor-like	yes	AAB23277	189, 190
			Zea m 12	14.2	14.1	n.d.	4.97–4.94	no	profilin	yes	P35081-3	155
<i>Dactylis glomerata</i>	orchard grass, cocksfoot grass	Poo	Dac g 1	31–33	26.1	5.5–6.4, 10	5.91	yes ¹	β-expansin	yes	CAD20406	18, 191
			Dac g 2	10–15	11.0, 10.8	5.1–5.5	5.3, 4.6	no		yes	2103117A, CAA10345	32, 64, 188
			Dac g 3	14–15	10.9	9	8.90	no		yes	P93124	32, 65
			Dac g 4	59–67	–	9–10.4	–	n.d.		no	–	32, 77
			Dac g 5	26–29	26.3	6.1	5.61	no		no	CAD20402-5	94, 116, 192
<i>Festuca pratensis</i>	meadow fescue	Poo	Fes p 4	60	–	8.7–9.1	–	yes		no	–	85
<i>Holcus lanatus</i>	velvet grass	Poo	Hol l 1	34 ³	26.1	4.6–5.2	5.53	yes	β-expansin	yes	P43216	9, 43
			Hol l 2	n.d.	11.2	n.d.	4.64	no		yes	CAA10347	188
			Hol l 5	30	26.2	4.7–6.2	6.01	no		yes	CAB10765-6	101, 109
<i>Hordeum vulgare</i>	barley	Poo	Hor v 9 (group 5)	30	30.1	n.d.	9.11	n.d.		yes	AAB41585	102
<i>Lolium perenne</i>	rye grass	Poo	Lol p 1	31–34 ³	26.1, 26.2	5.1–6.0	5.40, 5.57	5%	β-expansin	yes	P14946	19, 29, 36, 37
			Lol p 2	10–11	10.8	4.6–5.2	5.25	no		yes	P14947	19, 29, 61
			Lol p 3	10–11	10.9	9.0–9.4	7.86	no		no	P14948	19, 62
			Lol p 4	60–67	–	9.9	–	12–17%		no	–	74
			Lol p 5	25–34	–	5–8.3, >10	–	no		–	–	93, 97, 101
			Lol p 5a	–	29.1	–	10.20	no		yes	Q40240	103
			Lol p 5b	–	31.2	–	6.03	no		yes	Q40237	113
			Lol p 5c	–	27.5	–	5.11	no		yes	CAB64344	119
			Lol p 10	12	–	n.d.	–	n.d.	cytochrome c	no	–	139
			Lol p 11	18	14.9	5–6	5.1	8%	soybean trypsin inhibitor-like	yes	A54002	142
<i>Phalaris aquatica</i>	canary grass	Poo	Pha a 1	34	25.9	n.d.	5.27	yes ¹	β-expansin	yes	Q41260	41, 193
			Pha a 5	n.d.	31.2	n.d.	9.7	no		yes	P56164-7	41
<i>Phleum pratense</i>	timothy grass	Poo	Phl p 1	33–37 ³	26.2	6.2–8.5	6.16	5%	β-expansin	yes	P43213	31, 40, 59
			Phl p 2	11–12	10.8	4.7	4.64	no		yes	P43214	60
			Phl p 3	12	–	9	–	no		no	–	84
			Phl p 4	55–60 ²	–	9.0–9.5	–	10%		no	–	59, 75, 79
			Phl p 5	29–38	–	4.2–7.5	–	no		–	–	59, 99, 104
			Phl p 5a	–	28.7	–	6.89	no		yes	Q40960	98
			Phl p 5b	–	26.1	–	5.99	no		yes	Q40963	118
			Phl p 6	13	11.8	4.3–5.5	5.56	no		yes	P43215	59, 125
			Phl p 7	6	8.6	n.d.	4.19	no		yes	O82040	133
			Phl p 11	20	15.8	n.d.	4.96	yes ¹	soybean trypsin inhibitor-like	yes	AAN32987	144
			Phl p 12	14	14.2	4.4–4.9	5.07	no	profilin	yes	P35079	156, 159
			Phl p 13	55–60	41.7	6.5–7.4	7.83	n.d.	polygalacturonase-like	yes	CAB42886	163, 165
<i>Poa pratensis</i>	Kentucky bluegrass	Poo	Poa p 1	33–36	26.0	6.4, 9.1	6.40	yes ¹	β-expansin	yes	CAA10520	20, 188
			Poa p 2	n.d.	10.8	n.d.	4.64	no		yes	CAA10348	188
			Poa p 5	28–34	–	5.2–5.7, >9.5	–	n.d.		–	–	96, 117
			Poa p 5(a)	–	28.3–34.9	–	8.91–9.40	no		yes	P22284-6	96, 117
			Poa p 5(b)	–	27.2	–	8.59	no		yes	AAG42254	194
<i>Triticum aestivum</i>	wheat	Poo	Poa p 10	12	–	9.9	–	no	cytochrome c	no	–	137
			Tri a 2	n.d.	11.2	n.d.	4.64	no		yes	CAA10349	188

Subfamily was classified according to figure 1. Bam = Bambusoideae; Chl = Chloridoideae; Pan = Panicoideae; Poo = Pooideae. Allergen designation was according to the International Union of Immunological Societies Allergen Nomenclature Sub-Committee (www.allergen.org), where applicable. exp. = Experimentally analysed by electrophoresis; calc. = based on sequences using ProtParam on ExPASy; CHO = carbohydrates, i.e. presence (yes/no) or proportion of molecular weight; n.d. = not determined.

¹ Not demonstrated but suggested by the presence of a conserved glycosylation site and confirmed glycosylation of homologous protein(s).

² BG-60: 34 kD; Phl p 4: 38 kD by size exclusion chromatography.

³ Hol l 1: 27.6 kD; Lol p 1: 26.2–28.7 kD; Phl p 1: 28.1–28.4 kD by mass spectrometry.

tion or other additions which alter charge or mass. Genetically determined heterogeneity can be the result of allelic variation (gene divergence between individual plants) or the presence of a multigene family (two or more divergent genes in the same individual plant) encoding closely related proteins. Other mechanisms, such as alternative mRNA splicing and mRNA editing, may contribute to heterogeneity but are probably of minor importance.

Representatives of most allergen groups have been cloned and produced as defined recombinant proteins that may be used as tools to study cellular and molecular aspects of allergic sensitization and disease. Other recent developments are the use of recombinant allergens in diagnostic tests for allergy and the design of modified versions for novel therapeutic approaches.

Group 1

Members of the group 1 grass pollen allergens are recognized as the most prominent allergenic determinants in grass pollen extracts. About 90% of grass pollen-allergic individuals display IgE antibody reactivity to group 1 allergens, and in several species, this component has been shown to account for a considerable part of the specific IgE binding to the pollen extract [13–17].

Group 1 allergens are glycoproteins in a reported size range of 31–35 kD, as determined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), and in most cases slightly acidic [9, 18–24] (table 1). Localization studies have indicated their presence both in the cytoplasm and the outer wall layer (exine) of the pollen grain [25–28].

Isoform variation of group 1 allergens has been demonstrated in several species with respect to the isoelectric point, apparent molecular weight and/or amino acid sequence [9, 14, 20, 29–34]. Using two-dimensional (2-D) PAGE, followed by blotting and immunodetection, up to 10 spots corresponding to group 1 allergens have been observed in pollen extracts from several species [9, 30, 33, 35]. N-terminal microsequencing of Phl p 1 isoforms revealed identical 20-residue sequences of isoforms with the same molecular weight but different isoelectric points and divergent sequences of isoforms with different molecular weights [35].

Group 1 allergens have so far been cloned from at least ten species [15, 36–43] (table 1). Theoretical translations of cDNA sequences predict proteins of some 240 amino acid residues (excluding a signal peptide), with molecular weights of about 26 kD. Slightly higher mass determina-

tions, i.e. 26–28 kD, have been experimentally obtained using mass spectrometry [17, 34], consistent with the presence of glycan groups on these proteins. All group 1 allergens examined to date have an N-glycosylation site at amino acid position 9 [15, 19, 22, 23, 30, 42, 43] and carry glycans corresponding to 4–5% of their molecular weight [19, 44, 45]. In SDS-PAGE, the migration rate of group 1 allergens is somewhat lower than would be predicted from their molecular weight, which may in part be due to their glycosylation [34]. Group 1 allergens contain seven conserved cysteine residues, located mainly in the N-terminal half of the polypeptide.

Other modifications present in group 1 allergens include hydroxyproline residues at positions 5 and 8 in most species [22, 23, 30, 46] and possible phosphorylation at one or several of seven serine and threonine residues located within potential phosphorylation sites [23, 47].

Group 1 allergens show 60–70% sequence identity to expansins [48], a family of proteins involved in cell wall loosening and extension in plants through their activity of unlocking wall microfibril and matrix polysaccharide structures [49]. In a functional assay, group 1 pollen allergens from *P. pratense*, *Lolium perenne* and *Zea mays* have indeed been found to possess expansin-like activity, and a functional role of group 1 allergens in facilitating pollen tube growth through the female flower tissue upon germination has been suggested [48, 50]. The family of expansins has been divided into α - and β -expansins; the latter including group 1 grass pollen allergens as well as members expressed in vegetative tissues [48].

Production of group 1 allergens as recombinant proteins has proven difficult [42, 47]. Expression of Phl p 1 in *Escherichia coli* has been found to generate an insoluble product which can be refolded and kept in solution only in the presence of reducing agent at a relatively high concentration [unpubl. observations]. While the *E. coli*-expressed protein is clearly misfolded, a significant part of its IgE-binding structures are nevertheless preserved or restored, as evidenced by its activity as a solid-phase assay reagent [51–54]. Expression of Cyn d 1 in the yeast *Pichia pastoris* has been reported to result in secretion of a heavily glycosylated, soluble and immunologically active recombinant allergen [42]. Efforts to produce Phl p 1 in *P. pastoris* and insect cells have so far resulted in low yields of an unstable, hyperglycosylated protein product [34, 55, 56]. It appears that no generally applicable system for efficient production of group 1 allergens has yet been devised [47].

Group 2 and Group 3

Group 2 and group 3 grass pollen allergens were identified as a minor allergenic fraction in pollen extract of *L. perenne* more than 30 years ago [57, 58], and subsequently in *P. pratense* [59, 60]. Lol p 2 and Lol p 3 were purified and their complete amino acid sequence determined by Ansari et al. [61, 62]. Group 2 allergens have since been cloned from *L. perenne* [63], *Dactylis glomerata* [64], *P. pratense* [60] and several other species (table 1). The only group 3 allergen cloned to date is that of *D. glomerata* [65].

Groups 2 and 3 comprise 10- to 12-kD non-glycosylated proteins of 95–98 amino acid residues [19, 58, 60–62, 64, 65] which exhibit 85–90% sequence identity between species. The sequence identities between the group 2 and group 3 allergens of *L. perenne* and *D. glomerata* are 60 and 64%, respectively. The two groups differ considerably in net charge, members of group 2 being acidic, with observed isoelectric points of 4.6–5.5, while group 3 allergens are basic proteins [19, 32].

Group 2 and 3 allergens share a high degree of sequence homology (40% identity, 60–70% similarity) with the C-terminal part of group 1 allergens [61, 62, 66], suggesting a common origin by gene duplication [64]. Their sequence homology would indicate that group 1, 2 and 3 allergens may assume the same tertiary fold [67], and the C-terminal part of Lol p 1 has been modeled on the known structure of Phl p 2 [66]. Nevertheless, the sequence of group 2 allergens and the corresponding part of group 1 allergens are sufficiently divergent to confer different surface properties and more or less distinct antibody recognition [67]. Cross-reactivity between group 1 and group 2 or 3 allergens has been demonstrated for rabbit antibodies and human IgG by inhibition experiments [65, 68, 69], but not for human IgE antibodies [60, 68]. In contrast, significant cross-reactivity for human IgE was found to exist between Lol p 2 and Lol p 3 [68].

Members of groups 2 and 3 have been successfully expressed in *E. coli* [60, 63–65], and both the crystal and solution structures of recombinant (r)Phl p 2 have been determined [67, 70]. The molecule was found to be ellipsoid in shape and exhibit a stable 9-stranded β -sandwich resembling an Ig-fold, but without the specific features of the core packing observed in the immunoglobulin family. A C-terminal strand of Phl p 2 is not present in the Ig family but is shared with the C2 domain of some proteins involved in transmembrane signaling [71]. However, Phl p 2 lacks the Ca^{2+} -binding motif characteristic of C2

domains, suggesting that group 2 allergens represent a distinct structural family.

In several studies, the prevalence of IgE antibody reactivity to group 2 allergens among grass pollen-sensitized subjects has been found to lie in the range of 40–60%. However, the prevalence varies significantly between reports [54, 60, 72, 73], possibly as a result of differences between study populations or patient selection criteria [73].

Group 4

Group 4 grass pollen allergens were originally identified as 50- to 67-kD, highly basic, IgE-binding glycoproteins in *P. pratense*, *L. perenne* and *D. glomerata* [32, 59, 74]. Since up to 80% of grass pollen-sensitized individuals show IgE reactivity to group 4 allergens, they clearly qualify as major allergens [75–77]. Group 4 homologues have been detected by immunoblotting with monoclonal antibodies in all species studied so far, also outside the Pooideae subfamily [78–81]. They carry 10–15% carbohydrates and, although not yet demonstrated, it is likely that the IgE response to group 4 allergens is in part directed to the carbohydrate structures of the protein.

Taking advantage of their size and high isoelectric point, group 4 allergens can be efficiently purified using a combination of anion exchange chromatography and gel filtration [82]. Isolated group 4 allergens migrate as a broad, seemingly heterogeneous band on PAGE gels [83–85]. The group 4 allergen of *Festuca pratensis* was resolved into four spots in 2-D PAGE, of which three showed antibody binding activity [85]. It is not yet known whether the observed heterogeneity represents differences in modification or processing, allelic variants or multiple gene products, or a combination of these. Despite considerable efforts, cloning of a group 4 grass pollen allergen has so far not been reported.

Attempts to obtain amino acid sequences from group 4 allergens have shown that they are N-terminally blocked and resistant to trypsin digestion [77, 79]. Circular dichroism analysis demonstrated that Phl p 4 contains both α -helical and β -sheet structures, and, in contrast to several other allergens studied, Phl p 4 does not refold upon thermal denaturation [82]. Immunogold electron microscopy studies have indicated that Phl p 4 may be present both in the cell wall and the cytoplasm [86].

Extract of *C. dactylon* pollen has been shown to contain a high-molecular-weight glycoprotein component, BG-60, which binds IgE from and elicits skin prick test (SPT) reactions in a majority of grass allergic patients [87, 88]. The

carbohydrate moieties were found to contain $\alpha(1,3)$ -linked fucose residues on branched mannose structures, largely lacking the xylose residue common in other N-linked glycans from grass pollen [89]. Similar structures were subsequently identified in ragweed pollen proteins but have so far not been found in grasses other than *C. dactylon* [90].

Limited sequential proteolysis, circular dichroism experiments and peptide sequencing were used to study the structure of BG-60 [91]. The results suggested a three-domain configuration, of which the short N-terminal domain conferred compactness, thermal stability and protease resistance to the protein. A total of 180 residues of peptide sequence was determined, which showed similarity (40% identity) to plant berberine bridge enzymes, flavoenzymes involved in the biosynthesis of antimicrobial alkaloids. Spectral and fluorescence analysis of BG-60 indicated the presence of an FAD molecule attached to the central 35-kD domain, at a stoichiometry of about 1:1, and retention of the FAD in SDS-PAGE suggested a covalent attachment. This finding suggested that BG-60 is a pollen flavoenzyme, although its enzymatic function remains to be established.

The fact that an internal peptide sequence from Dac g 4, determined by Leduc-Brodard et al. [77], exhibits similarity to berberine bridge enzymes establishes a link to BG-60 and suggests that group 4 allergens of Pooideae may also be flavoenzymes and that BG-60 belongs to the group 4 allergens.

Group 5

Group 5 grass pollen allergens have been identified in many members of the Pooideae subfamily but not outside this taxon [10, 15, 81, 83, 92, 93]. Together with group 1 allergens, they dominate the allergenicity of grass pollen in species where they exist. Reported prevalences of sensitization to group 5 allergens range between 65 and 85% among populations of grass pollen-allergic individuals from temperate regions [9, 54, 92, 94–98].

Despite their apparent phylogenetic confinement, more diversity has been found within group 5 allergens than within group 1 allergens [3, 9, 93]. Group 5 allergens are similar in size to group 1 allergens, migrating in the 27- to 33-kD interval in SDS-PAGE. Isoelectric points between 4.2 and 9 have been reported for different members of the group, but most lie within the narrower range of neutral to slightly acidic (table 1) [9, 15, 83, 92, 97, 99–102]. Some basic members were originally designated group IX, but were later merged into group 5 because of

their immunological and structural similarity [93, 95, 96, 103]. No N-linked glycosylation has been reported to occur in group 5 allergens, but they contain several hydroxylated proline residues [9, 100, 101, 104, 105], two of which are at positions 7 and 10 from the N-terminus [97, 99, 104].

Group 5 allergens from at least seven species have been cloned and characterized to date (table 1). From most of these species, multiple sequence variants have been reported, and considerable amino acid sequence variation exists within the group as a whole. Two main isoforms have been discerned in *P. pratense* and *L. perenne*, denoted 'a' and 'b'. Reported variants of Phl p 5a and Phl p 5b from *P. pratense* show 25–30% amino acid sequence divergence and two distinctive insertions/deletions of 14–15 residues each in the N-terminal half of the protein, with Phl p 5a containing the additional residues in both cases. Phl p 5a is therefore slightly larger than Phl p 5b and it is also more basic [99]. One of the insertion/deletion differences can be seen also in comparisons of group 5 variants from *L. perenne*, although in this species, it appears that the naming of the two isoforms has in some cases been reversed as compared to *P. pratense*, which may cause confusion.

The overall amino acid sequence identity among known group 5 allergens from different species ranges between approximately 55 and 85%. Although many show preferential homology to one or the other of Phl p 5a and Phl p 5b, the extent and character of the sequence divergence within the protein family is such that the bipartite division into 'a' and 'b' isoforms does not seem fully applicable to members across the entire Pooideae subfamily.

Sequence analysis of group 5 cDNA clones predicts in most cases proteins made up of 260–300 residues and molecular weights of 26–31 kD. They contain no consensus site for N-linked glycosylation but show a distinctly skewed amino acid composition. Alanine constitutes up to 30% of the amino acid content, while the representation of methionine, cysteine, histidine and tryptophan is unusually low. Group 5 allergens have been located to the surface of amyloplasts and the cytosol [11, 26, 27, 103].

The structures of the C-terminal part of Phl p 5b and the N-terminal part of Phl p 5a were recently resolved by X-ray crystallography and nuclear magnetic resonance spectroscopy, respectively [106, 107]. Each protein fragment was found to form a folded globular domain consisting of a 4-helix bundle in a topology resembling that of cytochrome c. The crystal structure of the Phl p 5b fragment appeared as a dimer, covalently joined by a disulfide

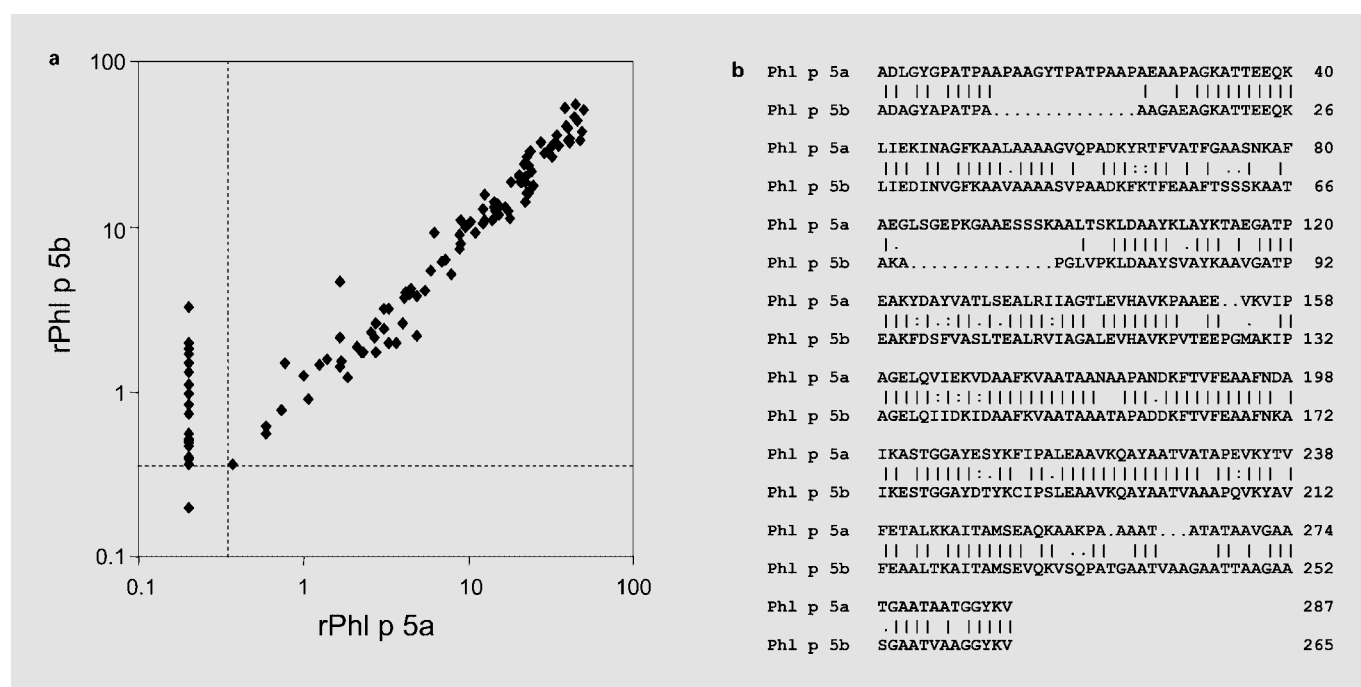


Fig. 3. Comparison of IgE-binding capacity and amino acid sequences of Phl p 5 isoforms. a Concentrations of specific IgE to rPhl p 5a and rPhl p 5b were plotted against each other. One hundred sera showed IgE reactivity to both isoforms, while 19 showed IgE binding to rPhl p 5b but not to rPhl p 5a. Values below the 0.35 kU_A/l cutoff level were considered negative and set to 0.2 kU_A/l. b Aligned amino

acid sequences of Phl p 5a (Acc. No. S37400) and Phl p 5b (Acc. No. AAC25995). Vertical lines indicate amino acid identity, while colons and periods denote strong and weak conservation of amino acid characteristics, respectively. Gaps are introduced to allow alignment across regions of insertions/deletions.

bridge between the two Cys²⁰⁵ residues. Phl p 5a contains no cysteine residue in its C-terminal part and would therefore not be able to dimerize by the same mechanism. Interestingly, we have observed that rPhl p 5b tends to dimerize in solution, whereas rPhl p 5a behaves as a stable monomer in gel filtration and non-reducing SDS-PAGE [unpubl. observations].

In an allergen group as diverse as group 5, it becomes relevant to examine whether allergenic differences exist. Somewhat contradictory results have been reported; some studies suggest that the 'a' and 'b' isoforms of Phl p 5 are immunologically equivalent [108, 109], while others show that both cellular [110–112] and humoral [101, 113–116] responses differ between the two isoforms.

Figure 3 shows a comparison of serum IgE antibody reactivity to rPhl p 5a and rPhl p 5b in a population of 150 grass pollen-sensitized individuals. While homologous allergens with conserved structure are usually highly cross-reactive (even if the observed intensity of antibody binding may differ), the two Phl p 5 isoforms show a markedly distinctive pattern of binding. IgE reactivity to rPhl p 5a

was present in 100 of the sera examined, and in all of these, a comparable level of IgE binding to rPhl p 5b was found. In contrast, 19 of the 150 sera showed IgE binding to rPhl p 5b which was not accompanied by IgE reactivity to rPhl p 5a. The observation suggests that Phl p 5b contains at least one important epitope for IgE antibodies which is quantitatively missing from Phl p 5a. An intuitive anticipation that the slightly larger of the two isoforms, rather than the smaller, would contain additional epitopes does not appear to hold true in this case. The observation illustrates that careful selection of allergen isoforms may be important in diagnostic and other applications, and that guidance by experimental investigation is required.

Group 5 allergens have been produced as recombinant proteins in *E. coli* as well as *P. pastoris* [41, 95, 96, 98, 102, 103, 109, 113, 116–119]. Expression in *E. coli* yields high levels of soluble protein which can be purified to homogeneity using standard methods.

The functional and biological role of group 5 allergens is still unknown, and their amino acid sequence is not strongly suggestive in this respect. Bufer et al. [118, 120]

reported ribonuclease activity associated with rPhl p 5b, in particular its C-terminal part, and speculated on a possible role in pathogen defense. The activity was, however, very low, and corroborative observations from other laboratories have so far not been reported.

Group 6

Group 6 grass pollen allergens have been identified only in *P. pratense* and *Poa pratensis*, and they appear to be restricted to these genera [10, 121]. Phl p 6 from *P. pratense* was first observed as a protein copurifying with and cross-reactive to Phl p 5, and was referred to as Ag19 [59, 122]. It was found to be an acidic, non-glycosylated protein with a molecular weight of about 13 kD, and N-terminal sequencing revealed homology to an internal region of group 5 allergens [123]. In 2-D PAGE, Phl p 6 has been found to separate into two spots with different isoelectric points, 5.2 and 5.5 [124], but the nature of this charge heterogeneity has not been further investigated.

cDNA cloning of Phl p 6 confirmed the homology to group 5 allergens and demonstrated 60% sequence identity to the C-terminal of Phl p 5, with patches of over 90% identity [125]. Its sequence similarity to Phl p 5 and restricted occurrence suggests a genetic ancestry of Phl p 6 as a product of group 5 gene duplication. The cDNA sequence of Phl p 6 predicts a protein of 110 amino acid residues, including 23 alanines (21%) but no cysteine, a molecular weight of 11.8 kD and an isoelectric point of 5.6. It contains one consensus N-glycosylation site near the N-terminus which, as shown by analysis of the native protein, is not used [124].

Phl p 6 has been successfully produced as a recombinant protein using *E. coli* as an expression host [121]. Analysis of rPhl p 6 by circular dichroism spectroscopy revealed a predominantly α -helical secondary structure [121], which is in keeping with the recently established crystal structure of the homologous C-terminal part of Phl p 5b [107].

Phl p 6 has been shown to bind IgE antibodies from 60–70% of grass pollen-allergic patients [121, 123] and would thus qualify as a major allergen. Due to their similarity, it is possible that Phl p 5 and Phl p 6 boost the immune response to each other and that the IgE recognition of Phl p 6 may therefore in part represent cross-reactivity to Phl p 5. In fact, there is little evidence of IgE antibodies that bind uniquely to Phl p 6, even though preferential binding to Phl p 6 over Phl p 5 by antisera from Phl p 6-immunized mice has been demonstrated

[124]. In our laboratory, only one of 87 rPhl p 6-reactive human sera did not show IgE binding also to rPhl p 5a or rPhl p 5b, and the level of IgE to rPhl p 6 in this serum was very low, <0.7 kU/l of allergen-specific IgE (kU_A/l). In the other 86 sera, the determinations of IgE to rPhl p 6 and rPhl p 5 showed a clear correlation, although most sera contained a significantly lower level of IgE reactivity to rPhl p 6 [unpubl. observations].

It has been suggested that Phl p 6 may be less immunogenic than Phl p 5, drawing from the observation that 50% of monoclonal antibodies obtained after immunization of mice with crude timothy grass pollen extract were directed against Phl p 5 but none against Phl p 6. Isolated Phl p 6 did, however, elicit an immune response in mice [124].

Phl p 6 has been located to polysaccharide-containing cell wall precursor bodies (P-particles) in the pollen grain [121], and, by analogy to Lol p 5, it is possible that its association with particles that may be liberated upon pollen breakage enables the allergen to penetrate further into the airways and cause bronchial symptoms [126].

Group 7

Group 7 grass pollen allergens are a group of small proteins that were first identified through immunoscreening of a *C. dactylon* cDNA library [127, 128]. Homologous mRNAs were detected in pollen of 13 different grass species and corresponding proteins have been cloned from several non-grass species, including *Brassica* species [129], birch [130, 131] and olive [132]. Representatives from grasses have so far only been cloned from *C. dactylon* [127, 128] and *P. pratense* [133].

Sequence analysis of cDNA clones for Cyn d 7 and Phl p 7 predicted proteins of 80 and 78 amino acid residues, respectively, with molecular weights of 8.7–8.8 kD and isoelectric points of 4.1–4.2. Neither sequence contained a signal sequence for secretion or a consensus site for potential N-glycosylation [127, 128, 133]. Cyn d 7 and Phl p 7 contain one cysteine residue but lack both tryptophan and tyrosine residues and therefore exhibit no absorbance at 280 nm.

The Cyn d 7 and Phl p 7 sequences were found to comprise two domains, each containing a Ca²⁺-binding EF-hand motif. No additional significant homology to known proteins was found, and the group 7 allergens thereby defined a new class of 2-EF-hand proteins which have come to be referred to as polcalcins [132]. The structural motifs present in the group 7 allergens suggested the ability to bind calcium ion, and it was indeed found that IgE

binding to membrane-bound Cyn d 7 and Phl p 7 was enhanced in the presence of Ca^{2+} and inhibited by EGTA, which acts to sequester calcium ions [127, 128, 133].

Cyn d 7 and Phl p 7 have both been produced as recombinant allergens using *E. coli* expression and shown to exhibit Ca^{2+} -dependent IgE antibody binding [127, 128, 133]. Analysis of rPhl p 7 by circular dichroism spectroscopy demonstrated a Ca^{2+} -dependent conformational change and high thermal stability in the presence of calcium ion, and Ca^{2+} acted to restore the secondary structure of heat-denatured apo-Phl p 7. rPhl p 7 was further found to elicit histamine release from basophils and induce skin reactions in sensitized subjects [133].

The three-dimensional structure of Phl p 7 was recently resolved by X-ray crystallography at a resolution of 1.75 Å [134]. The structure revealed that Phl p 7 exists as an intertwined, head-to-tail dimer, with each monomer containing two Ca^{2+} -binding helix-loop-helix domains connected by a 3-residue hinge. In 'classical' (monomeric) 2-EF-hand proteins, the corresponding linker is 6–14 amino acids long, which allows intramolecular pairing of the two EF-hands and an overall globular shape. While this is not possible in Phl p 7 due to the short hinge, intermolecular domain pairing results in a spatial arrangement of the loops identical to that in monomeric 2-EF-hand proteins. The Phl p 7 dimer was found to form a barrel with a large hydrophobic cavity, suggesting a ligand-binding function rather than a Ca^{2+} -buffering function alone [134].

Size exclusion chromatography confirmed the dimer assembly of Ca^{2+} -bound rPhl p 7 in solution, at neutral as well as low pH (4.8) [134]. Also, the apo-form eluted as a dimer but with a reduced elution volume, indicating an extended shape as compared to the Ca^{2+} -bound form. Taken together with the circular dichroism data [133], it was concluded that the allergen exhibits a Ca^{2+} -dependent conformational switch between the closed Ca^{2+} -bound form and the open apo-form [134].

Group 7 allergens are clearly minor with respect to the prevalence and intensity of IgE antibody reactivity. About 10% of grass pollen-sensitized individuals have been found to exhibit an IgE response to rPhl p 7 [54]. In most cases, the concentration of Phl p 7-specific IgE antibodies is low, and sensitization mainly occurs in subjects with high levels of IgE in response to the whole pollen extract [unpubl. observations]. Nevertheless, rPhl p 7 has been found to possess powerful allergenic activity in vivo and ex vivo in sensitized subjects, causing intense skin reactions and efficient histamine release from basophils at very low concentrations [133], possibly as a result of doubling of the local epitope concentration due to its dimeri-

zation [134]. Immunoblot inhibition experiments have shown that 2-EF-hand pollen allergens from both mono- and dicotyledonous plants are highly cross-reactive [130, 131, 133, 135] and that they also share epitopes with other Ca^{2+} -binding EF-hand pollen proteins, such as Bet v 3 (3-EF-hand protein) from birch and Jun o 4 (4-EF-hand protein) from *Juniperus oxycedrus* (prickly juniper) [136].

Group 10

Ekramoddoullah et al. [137] reported that cytochrome c, a 12-kD basic heme-containing protein from *P. pratensis*, was able to bind IgE and elicit intradermal skin reactions in grass pollen-allergic patients. The protein was further purified from *L. perenne*, *P. pratensis* and *C. dactylon* [137–140] and characterized with respect to antigenic and allergenic activity. However, Ansari et al. [141] failed to detect IgE and IgG antibodies to cytochrome c, either before or after immunotherapy with grass pollen extract. Later, cytochrome c was detected by monoclonal antibodies as a contaminant in preparations of Dac g 3, which is of similar size and net charge, but was found to be poorly recognized by IgE from grass pollen-sensitized patients [65]. Taken together, the available evidence indicates that cytochrome c is not a relevant grass pollen allergen and certainly not an important one.

Group 11

The group 11 grass pollen allergens were first defined in 1995 by van Ree et al. [142], who isolated and characterized an 18-kD, IgE-binding glycoprotein from pollen of *L. perenne*. The protein was purified by affinity chromatography using a monoclonal antibody, and its amino acid sequence was determined from a set of isolated fragments generated by proteolytic cleavage. The Lol p 11 protein contained a single site for N-linked glycosylation at residue 24 and carried carbohydrate corresponding to 8% of its molecular mass. The glycan moiety was found to consist mainly of M0XF³ and MMXF³ structures, which are present also in other grass pollen glycoproteins [143]. In isoelectric focusing, Lol p 11 appeared as several charge isoforms between pI 5.0 and 6.0 [142].

The 134-residue sequence of Lol p 11 predicted a protein of 14.9 kD with structural similarity to soybean trypsin inhibitor. Further, Lol p 11 showed 34–51% sequence identity to a number of other known pollen proteins, including olive pollen allergen Ole e 1, maize pollen pro-

tein Zm13 and tomato pollen protein LAT52. Lol p 11 and these other pollen proteins all contain six cysteine residues in conserved positions. However, none of the pollen proteins contain the active site structure of the soybean trypsin inhibitor, and no inhibitory activity towards trypsin could be detected in Lol p 11 [142].

van Ree et al. [142] found specific IgE reactivity to purified Lol p 11 in 179 of 270 (66%) sera from grass pollen-sensitized individuals. Approximately one third of the Lol p 11-reactive sera also showed IgE binding to carbohydrate, and in about one quarter of those sera, the IgE reactivity to Lol p 11 and a grass pollen-derived carbohydrate reagent was of similar intensity, suggesting that a portion of the IgE response to Lol p 11 was directed to the glycan moiety of the allergen.

Cloning of the group 11 allergen from *P. pratense* was recently reported by Marknell DeWitt et al. [144]. The cDNA sequence predicted a protein of 143 amino acid residues with 95% sequence identity to Lol p 11. rPhl p 11 was expressed as a fusion protein in *E. coli*, purified and characterized immunologically. The recombinant allergen elicited a skin reaction and histamine release from basophils of sensitized individuals and bound IgE from 59 of 184 subjects (32%) sensitized to grass pollen.

Group 12

Group 12 grass pollen allergens are members of a conserved protein family, profilins, which is represented in all eukaryotic cells [145]. Profilin was first described as an allergen in birch [146, 147] and *L. perenne* [148] and shown to cross-react with homologues from other plants [148, 149] and even with human profilin [146].

Profilins are 14-kD acidic proteins which are involved in cytoskeleton dynamics by binding to and controlling the polymerization of actin [145]. They also exhibit binding to poly-L-proline, which can be efficiently exploited in affinity purification [148, 150]. Profilins have been found to be minor allergens in pollens of grass and non-grass species, as well as in plant-derived foods, showing IgE binding from some 15–30% of pollen-allergic individuals [12, 54, 151], although a higher prevalence has been reported in some study populations [152–154]. It has been suggested that although IgE cross-reactivity between pollen and vegetable foods is common, it may frequently not be associated with symptoms of food allergy [154]. The level of amino acid sequence identity among profilins from the plant kingdom is about 80% and higher, whereas pollen and mammalian profilins are 35–40% identical.

Grass pollen profilins have so far been cloned from four species: *Z. mays* [155], *P. pratense* [156], *C. dactylon* [157] and *Oryza sativa* [158]. The cDNA sequence of Phl p 12 (originally referred to as Phl p 11) predicts a protein of 131 amino acid residues with a molecular weight of 14.2 kD and an isoelectric point of 5.0. The sequence contains two cysteine residues but no secretion signal or consensus N-glycosylation site.

Limited isoform variation appears to exist in grass pollen profilins. Natural profilin purified from *P. pratense* pollen extract was found to resolve into at least 5 bands in the pI range of 4.4–4.9 upon isoelectric focusing. Sequence analysis of three Phl p 12 cDNA clones obtained through RT-PCR revealed only 10 nucleotide changes, of which two caused amino acid substitutions [159]. In maize, however, profilin was found to be encoded by a multigene family of which at least four members were expressed in pollen, as concluded from sequence analysis of cDNA clones which diverged at 26 amino acid positions in total [155, 160].

Group 12 grass pollen allergens from *P. pratense* and *C. dactylon* have been expressed in *E. coli* and the purified recombinant profilins were found to be comparable to the corresponding natural allergens with respect to binding of rabbit anti-profilin IgG and/or human IgE antibodies [157, 161].

The three-dimensional structures of profilins from a wide range of organisms have been solved, including that of Bet v 2 from birch pollen [70]. As the pollen profilins are highly homologous, the Bet v 2 structure may be considered to represent this group of allergens. The 2.4-Å crystal structure of Bet v 2 revealed an α/β -structure comprising a central six-stranded, antiparallel β -sheet and three α -helices located on the surface. The overall fold was similar to those of mammalian and lower eukaryote profilins, although the orientation of the N-terminal α -helix was found to be different. It was suggested that this feature may affect the interaction with proline-rich ligands, which bind to a hydrophobic surface patch nearby [70]. IgE screening of a random subfragment library identified epitopes mainly on the N- and C-terminal α -helices and on an exposed two-stranded central fragment [70, 162].

Group 13

Group 13 is the most recently discovered group of grass pollen allergens. While fractionating *P. pratense* extract using hydrophobic interaction chromatography,

Suck et al. [84] found that a flow-through fraction contained three main protein bands, which could be separated by gel filtration. The largest of these proteins, 55 kD by SDS-PAGE, bound IgE antibodies from a pool of grass pollen-reactive sera but neither of two monoclonal antibodies raised against the similarly sized Phl p 4. Thus, the 55-kD protein was clearly distinct from Phl p 4, which, additionally, was completely retained on the phenyl Sepharose column under the conditions used. Using purified Phl p 13 in a nitrocellulose strip-based assay, it was demonstrated that 154 of 306 serum samples (50%) from grass pollen-allergic subjects contained detectable IgE reactivity to the allergen [163]. In the same experiment, purified Phl p 1, Phl p 2/3 and Phl p 4 bound IgE antibodies from 96, 60 and 56% of the subjects, respectively.

Petersen et al. [164] examined the occurrence of group 13 allergens in 10 different grass species and found that they migrate as two bands at 55–60 kD, on either side of the group 4 bands in SDS-PAGE and, further, that they form 4 spots with isoelectric points between 6.5 and 7.4 in 2-D PAGE. In contrast to Phl p 4, which is stable and highly resistant to protease digestion [79], purified Phl p 13 was found to be rapidly cleaved by protease Glu-C [163] and to degrade spontaneously during 24 h of storage at 4°C [164]. When present in extract, on the other hand, Phl p 13 appeared stable at 4°C [164].

Phl p 13 was cloned by 3'-RACE (rapid amplification of cDNA ends) [165] using a forward primer based on the N-terminal sequence obtained from the purified protein [164]. Sequence analysis of four clones revealed an open reading frame of 394 amino acid residues with 10 polymorphic positions. The protein deduced from the cloned cDNA which was subsequently selected for expression had a predicted molecular weight of 42 kD and an isoelectric point of 7.5. It contained 16 cysteine residues and four potential N-glycosylation sites [165].

Database comparison of the Phl p 13 amino acid sequence revealed an identity of about 70% over 389 aligned residues to polygalacturonase from pollen of *Z. mays* [166]. Polygalacturonases are pectin-degrading enzymes involved in cell wall turnover or remodeling, and the pollen enzyme has been proposed to have a role in pollen germination and/or pollen tube growth [167].

Phl p 13 was expressed in *E. coli* as a hexahistidine-tagged protein and, by SDS-PAGE, the purified protein showed an apparent molecular weight of 58 kD. Immunological activity of membrane-bound recombinant protein was examined by IgE inhibition experiments, and it was found to partially reproduce the antibody binding of the natural allergen [165].

Glycosylation

Glycosylation is a major secondary modification of proteins and adds another dimension of structural diversity, beyond that determined by genetic variation. Two principal kinds of proteoglycans exist, N-linked and O-linked, which are formed by different enzymatic pathways and comprise different carbohydrate structures [168]. N-glycans have attracted more investigation than O-glycans during the past decade, and several pollen allergens have been found to carry N-linked glycans. The functional consequences of glycosylation and the diversity it confers are, however, still poorly understood.

N-glycosylation is a complex biosynthetic process which takes place in the secretory pathway. A precursor oligosaccharide is initially attached to an asparagine residue of an acceptor site and the carbohydrate structure is then formed by iterative and consecutive steps of trimming and assembly. N-glycosylation requires the tripeptide Asn-X-Ser/Thr in the polypeptide chain, where Asn is the attachment point and X may be any amino acid except proline or aspartic acid. N-glycans are commonly divided into three classes based on structure and complexity: high mannose type, hybrid type and complex type [169], although the separation of a fourth class, paucimannosidic type, has been suggested [170].

While most parts of the N-glycosylation process are common to all eukaryotes, plants and invertebrates possess additional activities which generate structures that are not present in mammalian glycans. N-linked carbohydrates containing these structures, $\beta(1,2)$ -xylose and $\alpha(1,3)$ -fucose (fig. 4), are immunogenic, and IgE antibody binding to plant and insect N-glycans was first reported by Aalberse et al. [171] over two decades ago. Sialic acid, which occurs as a terminal structure in mammalian N-glycans, has not been found in plants or invertebrates [169].

The content of protein-linked carbohydrates has been studied in a variety of allergen sources, including pollens and vegetable foods [45, 74, 75, 90, 142, 143, 172–176], and both $\alpha(1,3)$ -fucose and $\beta(1,2)$ -xylose have been shown to be major determinants for IgE binding to N-glycans [90, 143, 176–178]. This field of study has been the subject of recent reviews [168, 170, 179].

N-glycosylation is not a highly defined modification but a rich source of glycoprotein heterogeneity of different kinds [170]. A particular glycosylation site may either carry or lack a carbohydrate structure attached to it. Further, the glycan at a particular site in a protein may be heterogeneous with respect to the number of glycan side chains in

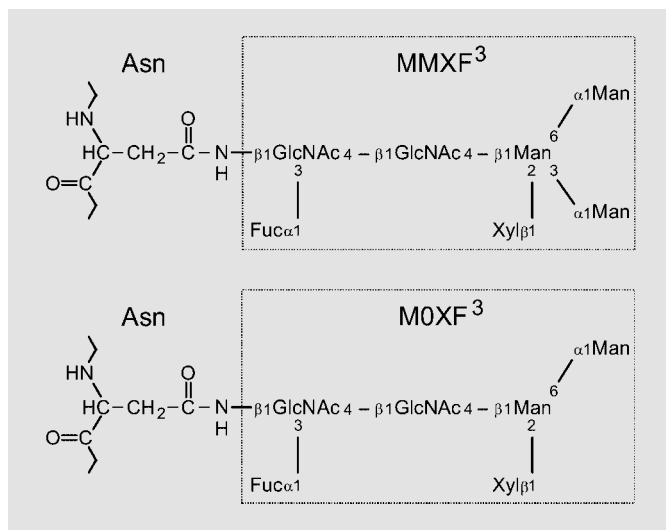


Fig. 4. N-linked carbohydrate structures detected in grass pollen allergens. The asparagine residue on the protein backbone is indicated and the carbohydrate moieties are boxed. Man = Mannose; Xyl = xylose; GlcNAc = N-acetylglucosamine; Fuc = fucose; Asn = asparagine.

the branched structure, as well as the extent of modification of the side chains.

Among grass pollen allergens, members of groups 1, 4 and 11 have been found to contain N-glycans, as discussed earlier in this text. The prevalence of carbohydrate-reactive IgE has not been systematically studied but appears to exist in 20–40% of grass pollen-allergic patients, however, very rarely without concomitant reactivity to peptidic epitopes [179, 180].

The fact that carbohydrate epitopes are highly cross-reactive and will bind specific antibodies regardless of the protein context has given rise to the term 'CCD' (cross-reactive carbohydrate determinants) [171] and stimulated a debate regarding the clinical significance of IgE directed to these structures. It has been demonstrated in a number of reports that glycan-mediated serological cross-reactivity is frequently not associated with clinical symptoms, particularly in the case of food allergens. These observations led to the notion that glycan epitopes and glycan-specific IgE antibodies would be clinically irrelevant [181–183], although other studies have shown that IgE reactivity to glycan epitopes may in some cases cause symptoms or elicit cellular reactions [173, 174, 184].

A plausible reconciliation of these seemingly contradictory results and conceptions was recently offered by Fötisch and Vieths [179], who suggested that the valency

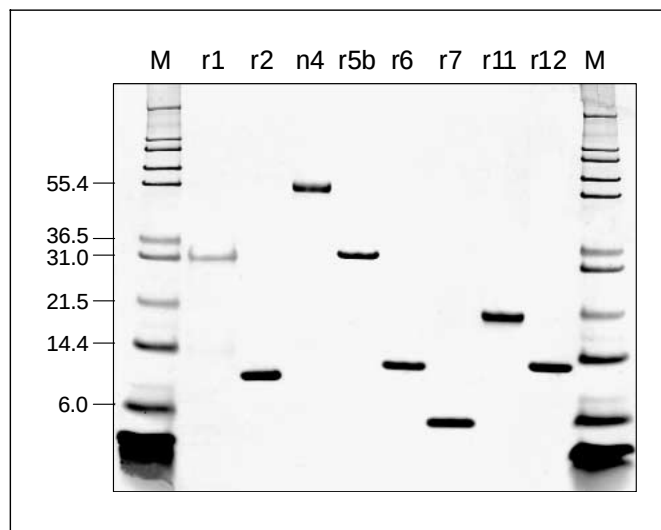


Fig. 5. Purified allergen components from *P. pratense*. Samples of *E. coli*-expressed rPhl p 1 (r1), rPhl p 2 (r2), rPhl p 5b (r5b), rPhl p 6 (r6), rPhl p 7 (r7), rPhl p 11 (r11), rPhl p 12 (r12) and natural Phl p 4 (n4) (0.1–0.3 µg) were separated on a 10 % NuPAGE® gel (Invitrogen, Carlsbad, Calif., USA) and stained with Coomassie brilliant blue. M = Molecular weight markers.

of the allergen's interaction with the IgE response, rather than its chemical nature, would be critical to the biological response. Accordingly, IgE binding to a glycan should not necessarily be less productive than binding to a peptidic epitope, provided that it acts in concert with IgE binding to other epitopes on the same molecule. No different from other epitope/IgE interactions, it would then contribute to the cross-linking of cell-bound IgE necessary for reaction. However, it is still possible that antibody binding to a glycan epitope is inherently weaker than antibody binding to a peptidic epitope and van Ree [185] has lately added the notion that a combination of valency and affinity of the interaction may better explain the observations that have been made. Regardless of their biological activity, glycans frequently contribute to antibody binding in assays for specific IgE and clearly represent a special case among allergenic structures since they may act as context-independent ('transplantable') epitopes.

Characteristics of the IgE Antibody Response to Grass Pollen Allergens

In allergic sensitization to grass pollen, IgE antibodies may be formed against any of the allergens described above. The sensitization pattern of allergic individuals

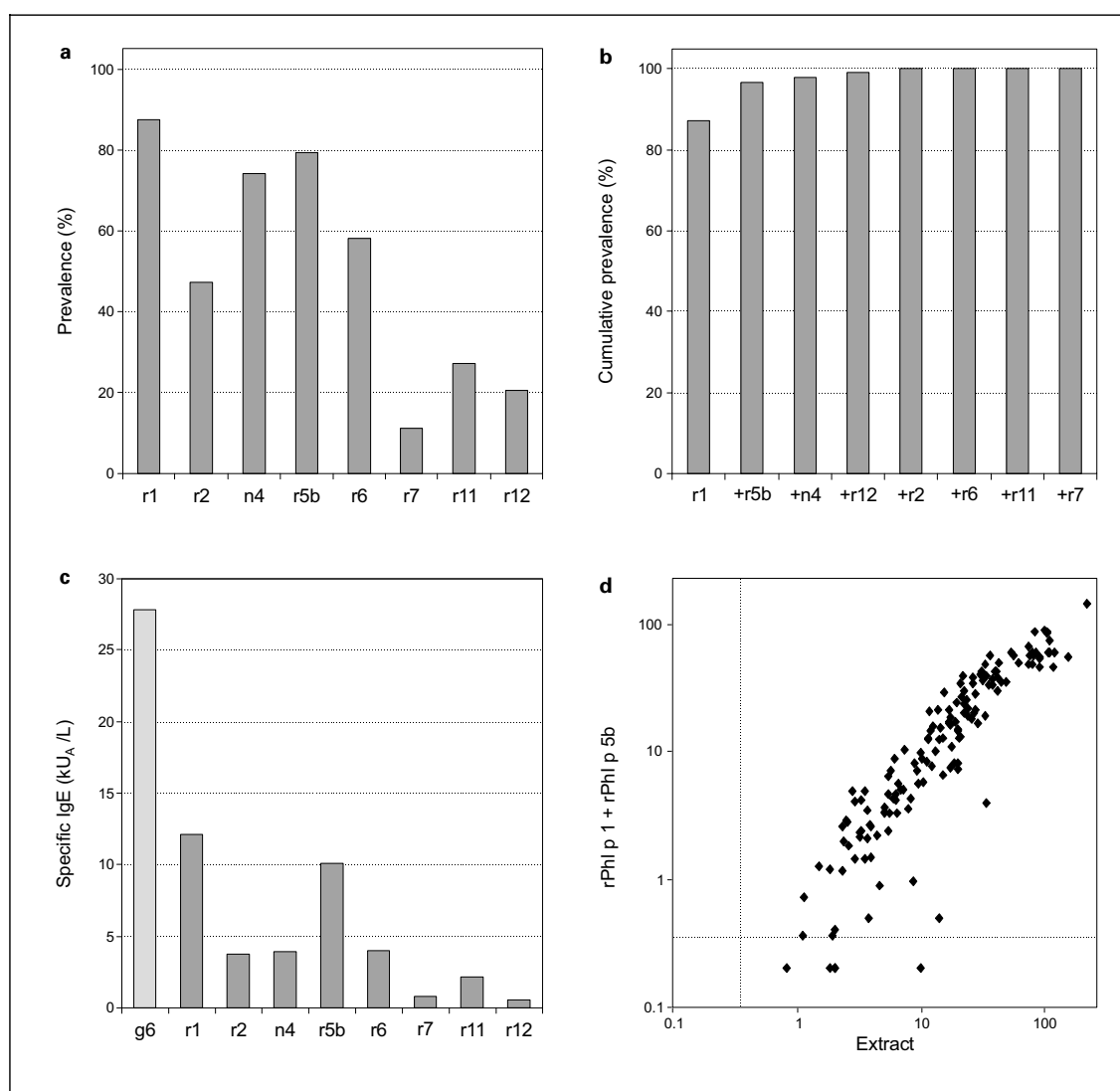


Fig. 6. Prevalence and magnitude of specific IgE to individual *P. pratense* allergen components. Concentrations of specific IgE to isolated allergens were measured in sera of 150 grass pollen-sensitized individuals. a The prevalence of specific IgE to individual *P. pratense* allergens. Concentrations ≥ 0.35 kU_A/l were considered positive. b Calculated cumulative prevalence of specific IgE to an increasing number of allergen components [rPhl p 1 alone, rPhl p (1 + 5b), rPhl

p (1 + 5b + 4), etc.], arranged in the order of incremental contribution to detection of additional subjects. c Concentration of specific IgE to natural *P. pratense* pollen extract (g6) and individual allergen components. d Concentration of specific IgE to a mixture of rPhl p 1 and rPhl p 5b, plotted against IgE values to natural *P. pratense* pollen extract. Values below the 0.35 kU_A/l cutoff level were considered negative and set to 0.2 kU_A/l.

may be studied by immunoblot analysis, although this method has the disadvantages of not allowing unambiguous identification of IgE-binding allergen bands and of being, at best, semiquantitative. Additionally, immunoblot analysis is a harsh method which may destroy certain epitopes and cause allergens to pass undetected by antibodies. The use of purified natural or recombinant allergens (fig. 5) in a quantitative assay system addresses all of

these problems and allows convenient, unambiguous and quantitative analysis of IgE reactivity profiles in patients.

Some population-level characteristics of grass pollen sensitization, determined using a panel of allergen component-specific IgE tests, are shown in figure 6. The population studied comprised 150 grass pollen-sensitized individuals with concentrations of specific IgE to *P. pratense* exceeding 0.7 kU_A/l. Analyses were performed with Im-

munoCAP tests specific for the panel of allergens shown in figure 5, i.e. rPhl p 1, 2, 5b, 6, 7, 11 and 12 and natural Phl p 4.

The prevalence of detectable IgE reactivity (≥ 0.35 kU_A/l) to each of these allergens is shown in figure 6a. In agreement with several previous reports, the group 1 and group 5 allergens were found to be the most important allergens, with a specific IgE prevalence of 87 and 80%, respectively, in this population. Natural Phl p 4 was recognized by IgE from 74% of the subjects, rPhl p 6 by 58% and rPhl p 2 by 47%. The representatives of groups 7, 11 and 12 bound IgE from far fewer subjects, i.e. 11, 27 and 21%, respectively.

The question then arises whether this set of allergen components will be sufficient to detect all cases of grass pollen sensitization and how the components should be most efficiently used to achieve that. Figure 6b shows a simulated experiment, based on the 1,200 data points described above, where the allergens are added to an assay one by one and used to test the same population of 150 subjects. Having the highest detection frequency among the allergens, rPhl p 1 forms the starting point and the other allergens are added in the order of incremental contribution to detection frequency in the study population. As expected, a saturation curve develops; by combining rPhl p 1 and rPhl p 5b, 145 of the 150 subjects (97%) are detected as positive, and by then adding natural Phl p 4, rPhl p 12 and rPhl p 2, another 2 + 2 + 1 subjects of the remaining 5 are detected, thereby reaching 100%.

When the quantitative aspect is considered, rather than merely positive/negative test results, the picture looks a little different. Figure 6c shows the average IgE levels for the grass pollen extract and each of the allergen components tested, as if a pool of all sera had been prepared and analyzed. Again, and even more so, rPhl p 1 and rPhl p 5b stand out as the most important allergens and together appear to account for about 80% of the specific IgE directed to the grass pollen extract. A striking difference to the prevalence figures illustrated in figure 6a is that the average level of IgE to natural Phl p 4 is relatively low; it appears to be the target of a frequent but low-magnitude IgE response. For the minor allergens (rPhl p 7, rPhl p 11 and rPhl p 12), this presentation of data tends to underrepresent the levels of IgE that can be found in individual patient sera.

If a conclusion from the simulated allergen combination shown in figure 6b is that rPhl p 1 and rPhl p 5b could be used together to form a test to detect most cases of relevant grass pollen sensitization, how would such a test compare to an extract-based test in a real experiment?

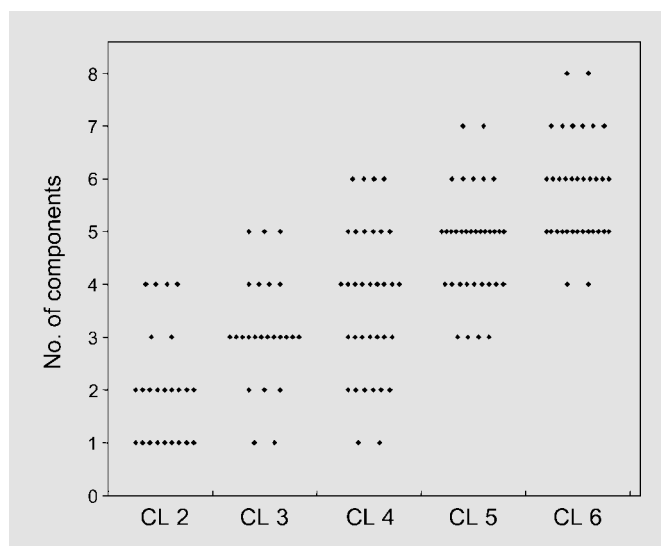


Fig. 7. Correlation between concentration of specific IgE to natural *P. pratense* pollen extract and the multiplicity of sensitization to individual allergen components. Sera of 150 grass pollen-sensitized subjects were analyzed. For each serum, the number of components to which IgE reactivity was detected was plotted against the concentration of specific IgE to the pollen extract, expressed as concentration class (CL) (class 2: 0.7–3.5 kU_A/l; class 3: 3.5–17.5 kU_A/l; class 4: 17.5–50 kU_A/l; class 5: 50–100 kU_A/l; class 6: >100 kU_A/l).

Figure 6d shows the results of just that experiment. As predicted from the data on the individual allergens and subjects, 145 sera tested positive and 5 were negative. Of the 145 positive sera, the quantitative determinations of specific IgE to rPhl p 1 + rPhl p 5 and to *P. pratense* extract were within a comparable range for 137 sera, and only 8 sera tested significantly lower on the recombinant allergen combination. These results illustrate quite clearly that even if grass pollen-allergic individuals display IgE reactivity to a range of allergen components, the bulk of allergenicity is contained in only two components. These data are in good agreement with the reported findings of other authors [7, 10].

Having tested all 150 serum samples with all allergens, it was found that most of all possible combinations of IgE reactivities were represented. Further, the entire range of multiplicity of sensitization was present, from sera that only reacted to a single component, to ones which reacted to all components. At the same time, the sera represented the entire concentration range of specific IgE to the grass pollen extract, from 0.7 to >100 kU_A/l. A reasonable assumption would be that the two parameters, multiplicity of sensitization and magnitude of specific IgE to the

whole extract, are related to each other. To examine this assumption, the number of allergens to which there was detectable IgE in each subject was plotted against the level of specific IgE to the grass pollen extract, expressed as concentration class (fig. 7).

As can be seen in figure 7, there is a very clear correlation between the number of IgE specificities detected and the level of anti-extract IgE. However, there is an extensive overlap between the classes, and from the level of specific IgE to the pollen extract, it is not possible to accurately predict the multiplicity of sensitization in an individual. Thus, to the extent that multiplicity of sensitization, as such, may carry important information (e.g. immunological disease activity, risk of developing clinical cross-reactions), test results obtained using the whole pollen extract would not be highly informative in this respect.

Diagnostic Use of Recombinant Grass Pollen Allergens

Recombinant allergens offer the possibility to dissect the allergic immune response and identify reactivity characteristics of potential clinical importance. One applica-

tion of this kind could be to distinguish patients who have a genuine and predominant grass pollen sensitization from those who are seropositive due to cross-reactive sensitization to other allergen sources, or have a highly mixed sensitization. The former would be characterized by a predominant IgE response to rPhl p 1 and/or rPhl p 5b, whereas the latter would show concomitant or exclusive sensitization to the minor allergen components. Such discrimination may serve to optimize the selection of patients suitable for specific grass pollen immunotherapy, which uses extracts standardized with respect to group 1 and group 5 allergens [186]. A significant aspect of using recombinant allergens produced in *E. coli* is that they lack any glycan modification present in the natural proteins, and measurements will therefore not be influenced by CCD reactivity.

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