

Pollen allergen homologues in barley and other crop species

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Summary

Pollen from 10 agricultural plant species was surveyed for the presence of proteins crossreactive with group I, group IV and group IX allergens. Barley (*Hordeum vulgare*), maize (*Zea mays*), rye (*Secale cereale*), triticale (*xTriticosecale cereale*), oats (*Avena sativa*), Canola (*Brassica napus*) and sunflower (*Helianthus annuus*) pollens contained numerous allergen cognate proteins. Northern blot analysis of barley pollen RNA revealed the presence of group I and group IX allergen transcripts. The barley pollen cDNA *hvp9742*, and three other cloned allergens: *phlenum protense* (*Phl p*) V, *Phl p* Va and *Lolium perenne* (*Lol p*) 1b, were demonstrated to have extensive nucleotide and amino acid sequence similarity to the *Poa p* IX isoallergens. It was concluded that *hvp9742* represents a *Poa p* IX isoallergen homologue expressed by barley pollen, and was therefore designated *Hor v* IX. It is further shown that the most highly conserved domains of all seven proteins, including *Hor v* IX, map to previously defined *Poa p* IX antibody binding epitopes.

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Introduction

Allergenic pollen proteins have been identified and cloned in a number of tree and grass species [1]. With the availability of primary sequence data [2] it is now possible to define important structural features of allergenic proteins by epitope mapping [3]. Recombinant allergens provide a reliable and inexhaustible supply of purified antigen which can be used for diagnosis and therapy of atopic allergies [4]. In addition, antibodies to purified antigens facilitate the analysis of potential antigens expressed by other wild plant species [5]. Since pollens of agricultural species represent a small fraction of the aeroallergenic substances routinely detected by mechanical surveys [6], they are widely assumed to be minor agents of pollinosis. Nevertheless, pollen of a number of agricultural species (including maize, oats,

sunflower, and cultivated rye) contain protein antigens which are weakly crossreactive with rye-grass allergen [7]. Indeed, patients with food allergies can be sensitive to pollen as well [8]. Food allergens from mustard, wheat, barley and rice seed have also been characterized [9–12]. Birch pollen profilin has recently been identified in both the pollen and vegetative tissues of many crop species [13]. Thus, it might be anticipated that the pollen of agricultural crops represent an additional source of allergenic proteins.

We examined the pollen of a variety of agricultural species, especially those of the *gramineae* family, for the presence of crossreactive group I, group IV and group IX allergenic proteins using antibodies (Table 1). We emphasize the analysis of one crop species, barley, by providing evidence that a barley pollen cDNA, *hvp9742* [16] is a group IX pollen allergen homologue. In addition, we show that the antibody binding epitopes of the *Poa p* IX isoallergen KBG60 correspond to the most highly conserved regions of the *Poa p* IX isoallergens (KBG 31, KBG41, KBG60), the group IX allergen cognate *hvp9742*, the timothy grass group V major allergens *Phl p* V and *Phl p* Va and the rye-grass group I allergen *Lol p* 1b.

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Table 1. Summary of pollen allergen properties and specific antibodies

Antibody	Allergen	Size (kDa)	pI	Species	Reference
MoAb 60	<i>Poa p</i> Ia	37	6.4	<i>Poa pratensis</i>	[14]
(Group I)	<i>Poa p</i> Ib	33	9.1	<i>Poa pratensis</i>	
MoAb 1	<i>Lol p</i> IV	57		<i>Lolium perenne</i>	[15]
(Group IV)					
anti-r8.3	<i>Poa p</i> IX				[2]
(Group IX)	KBG31	35	10.1	<i>Poa pratensis</i>	
	KBG41	30	9.7	<i>Poa pratensis</i>	
	KBG60	28	9.9	<i>Poa pratensis</i>	

Materials and methods

Pollen

Mature pollen of barley (*Hordeum vulgare*), wheat (*Triticum aestivum*), and Kentucky bluegrass (*Poa pratensis*) were initially obtained from Hollister-Stier Laboratory, (Mississauga, Ontario, Canada). Additional supplies of mature pollen of barley (*Hordeum vulgare* L. cv. Bonanza), wheat (*Triticum aestivum* L. cv. Katepwa), oats (*Avena sativa* L. cv. Rodney), rye (*Secale cereale* L. cv. Puma), and triticale (A290), were harvested from plants maintained under growth room conditions (18°C day, 15°C night, 16 h day, 50% relative humidity). Mature pollen samples of sunflower (*Helianthus annuus*), maize (*Zea mays*), Canola (*Brassica napus* L. cv. Westar) and flax (*Linum usitatissimum* L. cv. McGregor) were harvested from plants grown in the field.

Preparation of grass pollen proteins

Proteins were extracted from pollen as follows: pollen was frozen on a dry-ice/ethanol bath, freeze-dried, weighed and macerated with a glass/Teflon homogenizer in deionized distilled water until visibly disrupted, centrifuged for 15 min at 8000g and the supernatant was recovered. Protein content was determined by a micro-titre assay [17].

SDS-PAGE and immunodetection

Protein extracts were loaded onto 15% SDS-PAGE gels [18]. Proteins were either stained with silver [19] or electroblotted onto nitrocellulose [20]. Prior to immunodetection, blots were placed in blocking solution for 30 min. Blocking solution was 3% gelatin (w/v) in TBS (20 mM Tris, 500 mM NaCl, pH 7.5). For immunodetection, primary antibodies were diluted in TTBS (TBS plus 0.05% Tween-20, v/v) containing 1% gelatin (w/v). Blots

were rinsed three times with TTBS prior to addition of secondary antibody. Antigen-antibody conjugates were visualized by reaction to alkaline phosphatase-linked goat anti-mouse IgG, goat anti-rabbit IgG, or goat anti-human IgE secondary antibody (depending on the primary antibody used) diluted 1:7500 in TTBS containing 1% gelatin (w/v). Colour reaction of alkaline phosphatase was according to the manufacturer's instructions (Bio-Rad Laboratories).

Evaluation of human sera

Sera were obtained from 42 individuals attending Chedoke-McMaster Hospital, Hamilton, Ontario, who exhibited positive skin tests for grass pollen allergens and from 13 individuals who exhibited negative IgE to Kentucky blue grass (KBG) pollen extracts.

The cultivated barley pollen extracts were coated onto microtitre plates (Corning, NY, USA) in carbonate/bicarbonate buffer (pH 9.6) at 4°C overnight, followed by blocking with 1% bovine serum albumin (BSA) in PBS. They were then incubated with human sera at a dilution of 1:5 at room temperature overnight. Bound IgE was detected with alkaline phosphatase-conjugated goat anti-human IgE (1:1000, 2 h) (Sigma, St Louis, MO, USA). *p*-Nitrophenyl phosphate was used as substrate, and the absorbance was measured with an ELISA reader. All antibodies were diluted in PBST, 0.5% of BSA, and each incubation step was followed by washing with PBST. Cut-off values were determined by use of mean absorbances of 13 grass non-allergic individual sera plus two times standard deviation (cut-off value = $\bar{X} + 2\text{SD}$). The serum was considered positive if its absorbance was higher than the cut-off value.

Extraction of RNA from pollen

Five grams of pollen were hand ground in a dry-ice-chilled mortar and pestle. Pulverized pollen was

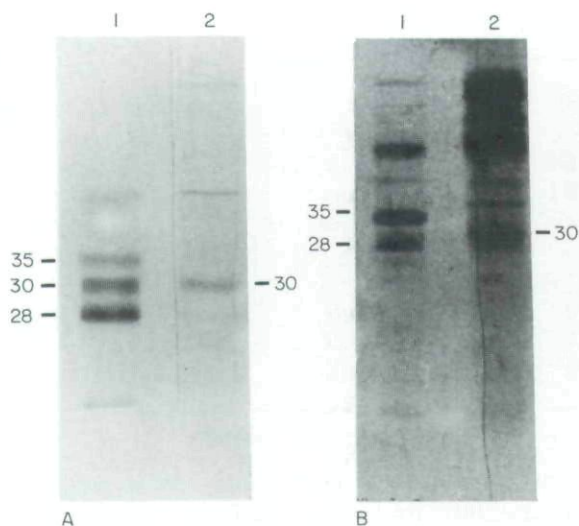


Fig. 1. Immunoblots of Kentucky bluegrass (lane 1) and barley (lane 2) pollen proteins. (A) anti-r8.3 antibody was presented at a dilution of 1:5000. (B) Human allergic serum IgE presented at a dilution of 1:10. 10 μ g of protein were separated by SDS-PAGE and electroblotted on nitrocellulose membranes. Membranes were probed with antibodies, followed by incubation with enzyme-conjugated second antibody and visualized with enzyme substrate.

placed in guanidinium iso-thiocyanate buffer [21] and blended with a PolytronTM tissue homogenizer for 30 s at 4°C. Cell debris was pelleted by centrifugation at 12 000 g for 20 min and the resulting supernatant subjected to cesium chloride buoyant density ultra-centrifugation [19]. Barley pollen poly(A⁺) RNA was affinity purified from total RNA by oligo(dT)-cellulose (Collaborative Research) chromatography [22].

Northern blot analysis

Total and poly(A⁺) RNA were electrophoresed in 1.5% agarose gels under denaturing conditions [23] and blotted onto Hybond-NTM (Amersham Ltd) nylon membranes. Radiolabelled probes were prepared by nick translation [24]. Hybridization [25] between probe and RNA was allowed to proceed for 12 h at 60–65°C, 6 X SSC (90 mM NaCl, 90 mM sodium citrate, pH 7.5), 5X Denhardt's solution (0.1% w/v each BSA, Ficoll, polyvinyl-pyrrolidone), 100 μ g/ml heat-denatured, sheared *Escherichia coli* chromosomal DNA, and 0.1% (w/v) sodium dodecyl sulphate (SDS). Post-hybridization washes were with 2X SSC, 0.1% SDS for 15 min followed by final washes. Autoradiographs of radioactive blots were made using Kodak X-OMAT AR film.

Sequence comparisons

Sequence analysis was performed using the Fristensky Sequence Analysis Package [26]. Additional DNA and protein alignments were produced with FASTA ver 1.5b, TFASTA, and LFATSA [27]. Sequence comparisons were made using data from GenBank rel. 79.0 and the Protein Information Resource (PIR) rel. 35. Consensus sequences were determined using the program PROFILE [28], and a sequence similarity score using a window of 10 amino acids was generated with the program PLOTSIM [29].

Results

In a preliminary experiment, barley pollen was tested for the presence of proteins capable of crossreacting with antibodies specific for group I, group IV and group IX pollen allergens (Table 1). In addition, human allergic serum (HAS), from a pool of sera of 11 individuals allergic to grass pollen known to contain IgEs for KBG pollen allergen was tested. Barley pollen proteins reacted with all four antibody preparations. Specifically, the 37 and 33 kDa group I allergens were present, the 57 kDa group IV allergen was present, one group IX isoallergen (30 kDa) was present, and a variety of IgE reactive proteins were present including one at 30 kDa. Figure 1 shows immunoblots with anti-r8.3 (Fig. 1A and HAS IgE (Fig. 1B).

³²P labelled c14.4, a potential group I cDNA clone [30], and c7.2, a fragment of the group IX cDNA clone KBG41 [31] were used to probe total and poly(A⁺) RNA isolated from Kentucky bluegrass and barley pollen. The c14.4 probe detected transcripts of 1.2 kb in both species, and the c7.2 probe detected transcripts of 1.5 kb in both species (Fig. 2). The signal strength of both transcripts in barley was weaker than Kentucky bluegrass under the same hybridization conditions. However, the signals in barley were clearly visible in poly(A⁺) RNA preparations.

To survey the pollen of agricultural species, proteins were extracted and tested by immunoblotting with group I, group IV, group IX, and HAS IgE. The results are summarized in Table 2. Generally, non-gramineous crop species possessed few proteins capable of crossreacting with the antibodies tested. However, all species reacted with at least one antibody, and all but wheat, *Brassica napus*, and *Linum usitatissimum* reacted with human allergic serum IgE. The crossreactivity of barley pollen extracts with sera from 13 grass non-allergic and 42 grass allergic individuals is illustrated in Fig. 2. Twenty-six out of 42 grass allergic individuals show specific IgE antibodies to cultivated barley. None of the sera

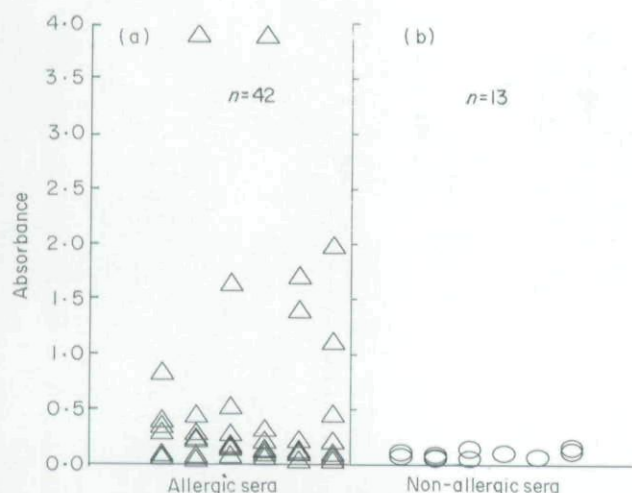


Fig. 2. Reactivity of barley pollen proteins with sera from (a) allergic ($n = 42$) and (b) non-allergic ($n = 13$) individuals.

from non-allergic individuals showed any appreciable reactivity.

We have identified a barley pollen cDNA clone [16], named *hvp9742*, which crosshybridizes to group IX isoallergen cDNA clones KBG31, KBG41 and KBG60. A sequence database search using *hvp9742* DNA revealed that in addition to the *Poa p* IX pollen isoallergens, three other cloned pollen allergens share substantial nucleotide (>50%) and amino acid (>60%) sequence similarity (*Phl p* V [32], *Phl p* Va [33] and *Lol p* Ib [34] with *hvp9742*. In addition, the nucleotide sequence of *hvp9742* was

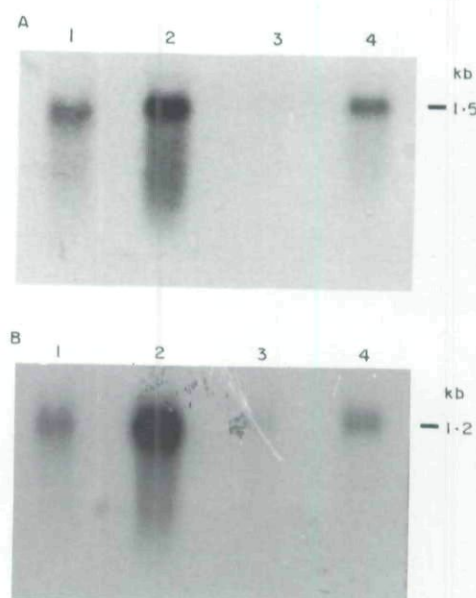


Fig. 3. Northern blot analysis of pollen RNAs; 10 μ g of *Poa pratensis* pollen total RNA (lane 1), 0.1 μ g Kentucky bluegrass pollen poly(A⁺)RNA (lane 2), 10 μ g barley pollen total RNA (lane 3) and 0.1 μ g barley pollen poly(A⁺) RNA (lane 4) probed ³²P-cDNA labelled by nick-translation.

more than 70% similar to KBG41. The putative polypeptide of *hvp9742* has a molecular weight of 30 kDa and an isoelectric point of 9.9, which is also very similar to KBG41 (KBG41 encodes a polypeptide of 30 kDa with an isoelectric point of 9.7). The polypeptide sequence of

Table 2. Allergen cognate proteins in various crop species

Species	Pollen allergen antibodies			
	Group I	Group IV	HAS IgE	Group IX
Gramineous				
<i>Poa pratensis</i>	33, 37 (57)	57	17, 28, 35, 59	28, 30, 35
<i>Hordeum vulgare</i>	33, 37	57	30, 42, 59, 85	30
<i>Zea mays</i>	17, 20, 24, 33	—	35	33, 46, 55
<i>Secale cereale</i>	33, 37	(24), 57, 69	35, 59, 85	30, 46, 69
<i>Triticale</i>	37	(24), 57, 69	35	30, 33, 46, 69
<i>Triticum aestivum</i>	(39)	—	—	—
<i>Avena sativa</i>	(24), 33, 57	(24), 57, 69	35, 85	24, 46, (55, 69)
Non-Gramineous				
<i>Brassica napus</i>	—	55, 65	—	55, 65
<i>Helianthus annuus</i>	46, 60, 69	24	17, 42, 85	30
<i>Linum usitatissimum</i>	—	46	—	—

Proteins from pollen of species listed were probed on western blots with anti-allergen antibodies listed in the column headings. Proteins are identified by molecular size (kDa). Proteins indicated in brackets are minor (faint) compared with others listed. Antibodies were presented at titres optimal for identification in *Poa pratensis*.

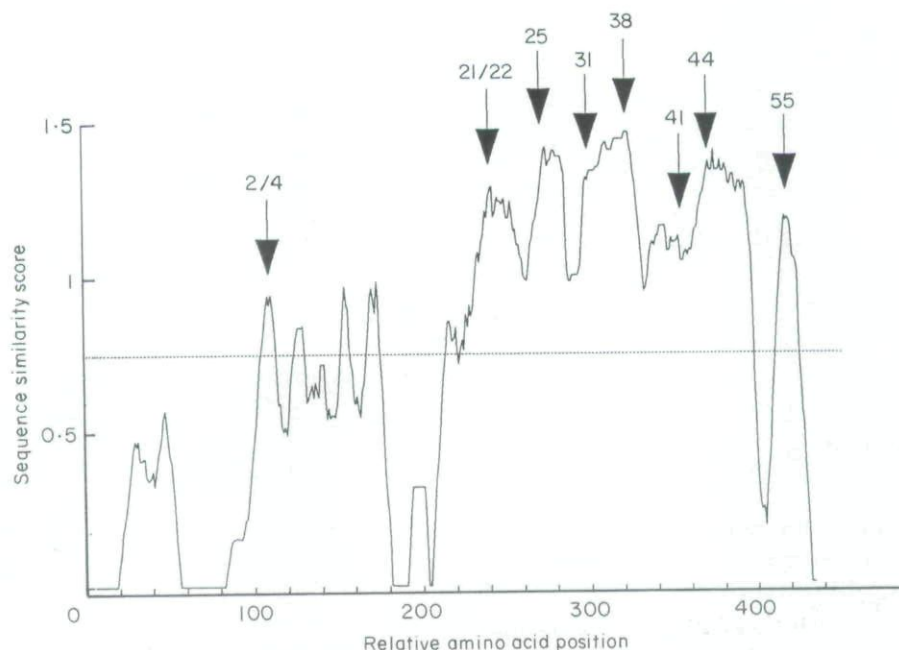


Fig. 4. Sequence similarity score of seven pollen allergens, (*Poa p* IX KBG31, *Poa p* IX KBG41, *Poa p* IX KBG60, *hvp*9742, *Phl p* V, *Phl p* Va, and *Lol p* Ib). The degree of sequence similarity (X-axis) is indicated for a sliding window of 10 amino acid residues. Highly conserved residues receive a high score, unconserved residues receive a low score. Amino acid position relative to the aligned consensus sequence is indicated (Y-axis). Arrows with numbers indicate the position of antibody binding epitopes mapped for KBG60 [3].

*hvp*9742 is 80% similar to KBG41 (and the other *Poa p* IX isoallergens).

By aligning the amino acid sequences of these pollen allergens, a grass pollen allergen consensus sequence was generated. The amino acid sequence similarity score using overlapping windows of 10 residues for the

pollen allergen genes (KBG31, KBG41, KBG60, *hvp*9742, *Phl p* V, *Phl p* Va, and *Lol p* Ib) relative to the consensus sequence was plotted (Fig. 4). The known antibody finding epitopes of KBG60 [3] appear to occur in regions where the pollen allergen proteins are most highly conserved. In fact, the consensus sequences of all

Table 3. Conservation of antibody binding epitopes in seven pollen allergens

Epitope number	<i>Poa p</i> IX (KBG60) Amino acid sequence [3]	Consensus amino acid sequence
2	SYGAPATPAA	(<u>G</u> /S)YG(<u>X</u>)PATPAA
4	PAAGYTPAAP	PAAGYTPAAP
21–22	YKLAY	YKLAY
25	KYDDYVALTS	KYDAYVATLS
31	EVKATPAGEL	EVKA(I/V)PAGEL
33–34	VDAAF	VDAAF
38	FTVFEEAFND	FTVFEEAFND
41	TGGAYQSYKF	TGGAY(Q/E)SYKF
42	QSYFKFIPALE	(Q/E)SYKFIPALE
44	AAVKQSYAAT	AAVKQ(S/A)YAAT
55	AATGAATAAA	AATGAATAA(T/A)

*Variable residues are indicated in parentheses (i.e. G/S = glycine or serine), and 'X' indicates 'any amino acid'. Substitutions in the consensus relative to KBG60 are doubly underlined.

corresponding antibody binding epitopes of KBG60 were 90–100% similar in all seven pollen allergens (Table 3). Of the six substitutions present in the consensus epitopes, all were conservative.

Discussion

Immunoblotting experiments using group I, group IV, group IX and HAS IgE antibodies demonstrated that barley pollen expresses proteins capable of crossreacting with anti-allergen antibodies. The crossreactive proteins are exactly the same molecular weight as those found in Kentucky bluegrass. Northern blotting experiments showed that messenger RNAs homologous to group I cDNA (c14-4) and group IX cDNA (c7-2) are indeed expressed by barley pollen. In fact, c7-2 has been used subsequently to identify a number of barley pollen cDNAs [16]. One c7-2 selected barley pollen cDNA (hvp9742) encodes a polypeptide 80% similar to the *Poa p IX* isoallergen KBG41. The immunoblotting and northern blotting experiments reported herein, in combination with the high nucleotide and amino acid sequence similarity of hvp9742 to KBG41, suggest that hvp9742 encodes a *Poa p IX* allergen cognate, which we designate *Hor v IX*.

It has previously been demonstrated by several investigators that monoclonal antibodies can be employed to identify crossreactive allergens in a variety of grass species [7,35]. Group IX proteins in wild grass species have been examined previously [5]. Now, we have extended the survey of potential pollen allergens to agricultural crop species. Immunoblotting experiments using group I, group IV, group IX and HAS IgE antibodies demonstrated that the pollen of a number of other gramineae and non-gramineae crop species express proteins which are candidate allergens. Crossreactive proteins ranged in size from 17 to 85 kDa. Sunflower contained unusually high molecular weight MoAb 60 reactive proteins (46, 60, and 69 kDa) compared with KBG, whereas it contained a single low molecular weight species of 24 kDa for MoAb 1, which normally identifies a 57 kDa protein in KBG. The 24 kDa protein was observed in a number of other species, including rye, triticale, and oats. The gramineous species of barley, maize, rye, triticale and oats contained the greatest number of reactive proteins (more than eight). Sunflower also contained a large number of antigenic proteins (eight). In the collection of gramineous species, only wheat showed low reactivity, with one protein reactive to MoAb 60 and none reactive to MoAb 1, anti-r8·3, and HAS IgE. This finding suggests that the allergens present in triticale, a hybrid of wheat (*Triticum aestivum*) and rye (*Secale cereale*) were contributed to by

the rye genome. Flax also had no proteins reactive with HAS IgE, anti-r8·3, and MoAb 60; and reacted only with MoAb 1. We conclude that the pollen of barley, maize, rye, triticale, oats, canola and sunflower represent a potential source of aeroallergenic proteins for individuals proximal to agricultural sites.

Poa p IX, *Hor v IX*, *Phl p V*, *Phl p Va* and *Lol p Ib* were shown to have striking amino acid sequence similarity. In addition, the regions of greatest similarity among these allergens (and the cognate *Hor v IX*) correspond exactly with the antibody binding epitopes mapped for KBG60. The conservation of total amino acid sequence concomitant to the location of antibody epitopes strongly suggests that all seven genes arose from a common ancestor. The exact nature of the evolutionary constraints on the antibody binding epitopes is presently unknown. Perhaps the sequences which produce good antibody binding sites are also important to the function of these pollen surface proteins. Two-dimensional polyacrylamide gel electrophoresis of proteins extracted from barley pollen at different stages of development shows that many group IX-like (i.e. having an apparent molecular weight close to 30 kDa and an isoelectric point greater than pH 9.0) species accumulate at maturity [36].

It is clear from our results that individuals with allergies to the commonly occurring grass pollens such as *Poa*, *Phleum*, and *Lolium* should be aware that there may be a significant occupational hazard when dealing with agricultural crops, which we have now shown to crossreact with proven grass pollen allergens. Our sequence similarity profile shows that the important epitopes are conserved among known allergens as well as the cognate found in barley, an agricultural crop.

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