ORIGINAL ARTICLE

Purification and characterization of *Parthenium hysterophorus* flower proteins that exhibit inherent immunological responses

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Abstract

Introduction: To insight the importance of immunoglobulin's (Ig) role in allergic reactions and to determine the mechanism of IgG in allergic responses. **Aim:** This study aimed at purifying the proteins from *Parthenium hysterophorus* flower and to check their allergic activity with IgG. **Materials and Methods:** Purification methods of phenol extraction for *P. hysterophorus* flower proteins and affinity chromatography for IgG purification were used. The purified allergens were characterized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), two-dimensional (2D)-electrophoresis, mass spectrometry, immunoblot, and IgE-ELISA. **Results:** SDS-PAGE analysis of pollens under reduced conditions revealed four proteins with molecular weights 44 kDa, 38 kDa, 18 kDa, and 23 kDa as the major allergens of *Parthenium* pollen with an isoelectric point of 10 by 2D-PAGE. Furthermore, cross-reactivity between allergens was investigated by IgG-ELISA, which was identified at 100 ng/ml concentration of IgG antibody. The result of Fourier transform infrared spectroscopy analysis confirmed the presence of alcohol and amides, whereas the mass spectra were matched by the National Center for Biotechnology Information/Blastp to find possible glycopeptides applying settings of GlycoMod search program. **Conclusion:** The research findings suggest that several proteins in the range of 18-40 kDa could be used as diagnostic markers for patients allergic to *P. hysterophorus*.

Key words: Allergen, congress grass, GlycoMod, *Parthenium hysterophorus,* pollens

INTRODUCTION

Parthenium hysterophorus, popularly known as congress grass, is a flowering plant in the family Asteraceae.[1] It is native to the American tropics and is a common invasive species in India, Australia, and certain parts of Africa. It first appeared as a contaminant in imported wheat after which it became an invasive species.[2] Pollen form *P. hysterophorus* has been reported as an important source of pollinosis in native of American tropics but also exposed in tropical and subtropical regions of the world.[3] The plant is known to produce allelopathic chemicals that have the ability to restrain crop plants and allergens that can affect humans and livestock. Moreover, it also regularly causes pollen allergies.[4] Major allergens from the Sorghum pollen grains have been referred to be in the range of 14-18 kDa which are small acidic proteins accountable for large number of allergic cross-reactivity.[5] The earlier observations suggest a close allergenic relationship between other grass plants.[6] Plant allergy is an immunoglobulin E (IgE) and IgG-mediated adverse reaction to plant proteins leading to symptoms such as mild erythema or rhinitis, to acute and possibly fatal anaphylactic shock.[7] The main

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allergen sources in plant allergy are flowers, grasses, trees, and weeds. About 2.3% of children and 1.4% of adults are affected by plant flower allergy.[8] In the human body, IgE is downstream from IgG, hence, IgE creating B-cells switch to IgG production, but the role of antiallergen IgG antibodies in allergy remains unclear.[9] This study discusses the possible hypothesis which can explain the association between IgG and IgE causing B-cells to become regulated. Purification of Igs is required for many applications in various fields of biotechnology. Plant-allergic patients have high allergen-specific IgG levels. However, the role of these antibodies in complex formation and their binding to B-cells is unknown.[10] Most papers deal with the purified Ig as starting material to check the responses toward plant allergens, food allergens, and dust allergens.[11]

MATERIALS AND METHODS

Purification of Grass Pollens from *P. hysterophorus*

P. hysterophorus plants were collected from K L University campus, Green Fields, Andhra Pradesh, India. The pollen from the flowers was rubbed under sterile conditions and homogenized with a mortar and pestle in 0.5 ml of extraction buffer (0.7 M sucrose, 0.5 M Tris, 30 mM HCI, 50 mM ethylenediaminetetraacetic acid, 0.1 M KCl, 2% [v/v] 2-mercaptoethanol, 2 mM phenylmethylsulfonyl fluoride and pH 8) and made up to 1.5ml with extraction buffer which was then incubated at 4°C for 10 min. Water saturated phenol was added in the ratio of 1:1 and after incubating for 10 min, allergens[12] were precipitated from the phenol phase by addition of 5 volumes of 0.1 M ammonium acetate in methanol and was incubated at −20°C overnight. The precipitate was washed three times with the ammonium acetate in methanol and once with acetone. The pellet was dried and solubilized, and all the samples were stored at −20°C. Desalting columns (Zeba spin, Thermo) were used to remove traces of salts from the protein.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) Analysis of Proteins

Purity of IgG and pollens was checked by 12% SDS-PAGE of reduced and nonreduced samples. For reduced gels, 2% β-mercaptoethanol, 160 mM dithiothreitol (DTT) along with SDS loading buffer was used and boiled at 100°C for 5 min.[13] Ezee blue staining reagent (direct stainer; Merck Biosciences) was used to visualize the protein bands. As a standard, broad range molecular-weight marker from Puregene was used. All samples and buffers used in this study were filtered with Microcon centrifugal filters to remove small particles and buffers were degassed before use. Protein determination was performed by the Bradford method, using bovine serum albumin as a standard.

Two-dimensional (2D) SDS-PAGE Analysis of Proteins

For 2D electrophoresis, proteins were analyzed on immobilized pH gradient strips pH 4-7 (Merck Biosciences) and 12% SDS-PAGE subsequently. For isoelectric focusing strips (IEF strips; Thermo), rehydration buffer consisting of 8 M urea, 4% CHAPS, 0.5% Triton X-100, 0.4% carrier ampholytes (Pharmalytes 3-10, Sigma), and 10 mM DTT, and trace of bromophenol blue was used.[14] Isoelectric focussing of pH gradient strips was performed on 2D-elpho system (Merck Biosciences) until the dye reaches the end. IEF strips were immediately transferred to equilibration buffer (6M urea, 2% (w/v) SDS, 30% (v/v) glycerol, 50 mM Tris, pH 8,6) with interval of 20 min for 2 times. The second dimension was carried out in Mini-Protean cell (Biorad) at 50 V for 30 min, followed by 100 V for 4 h. The gel was stained with Ezee Blue direct stainer and gels were scanned using UVI-Tech bioimager, and ImageMaster was used to remove artificial spots and dust particles.

Immunoblotting

Immunodetection of proteins in nitrocellulose membrane was achieved as described,[15] using 20 µg of protein on 12% polyacrylamide gel, and proteins were transferred to a nitrocellulose membrane; pore size of 0.3 µm with thickness of 10 µm (BioTrace, Pall Life Sciences) using semi-dry procedure with e-blotter (Genetix Biotech) at 50 V for 2 h. After blocking with 1% bovine serum albumin in Tris-buffered saline with Tween 20 (TBST) for 1 h, the membrane was incubated with primary antibody (Santa-Cruz) in 1:2000 dilutions (IgG affinity purified) at 40°C for 2 h. Immunoblots were developed with goat antihuman IgG-horse radish peroxidase conjugate (Santa-Cruz) and subjected to visualization.

Purification of IgG by Affinity Chromatography

Affinity chromatography was performed on protein A sepharose[16] (2 ml columns, Merck Biosciences). Equilibration buffer was 0.05 M sodium phosphate, pH 7.2, and elution buffer was 0.2 M glycine, pH 2.5. Column was washed with 0.1 M glycine buffer following with equilibration buffer. Flow rate was setup at 1 ml/min, serum samples were applied on the column by sample loop, and protein load was between 0.5 and 10 mg for analytical and preparative scale, respectively. Microcon centrifugal filters (Merck, MRCFOR30) were employed for high recovery of protein with concentration factor of <10X. Protein concentration was determined using a bicinchoninic acid[17] protein assay (Thermo Pierce, 23255).

High-performance Liquid Chromatography (HPLC) Analysis of Proteins

Extracts were analyzed on Shimadzu chromatography system using HPLC-Phenomenex 3000 pump (Schimadzu) Luna 5u

C18 column (250 × 4.6 mm).[18] Two buffers were prepared for a gradient type elution. Elution A: 0.1% trifluoroacetic acid (TFA) in 95:5 water/acetonitrile (ACN), elution B: 0.085% TFA in 75:20:5 ACN/PA/water. Owing to the gradient, a certain ratio for both buffer volumes was maintained and elution was performed with eight volume combinations, *viz*., (A: B) of (80:20) (70:30) (60:40) (50:50) (40:60) (30:70) (20:80) (10:90). ACN washes were done to free the column of any contaminants followed by equilibration with buffer and loading of the sample. The flow rate was adjusted to 1 mL/min, and the samples were detected using UV-Vis Abs.-Diode Array (PDA) @ 220 nm (22°C).

Determination of Allergen Binding Toward IgG by ELISA

To determine allergen specificity toward IgG, 96 welled plate (Affymetrix eBioscience) was coated with 10 µg/ml concentration of allergen and incubated at room temperature for 2 h or at 4°C for overnight. Simultaneously, pre-coated IgE plates (Affymetrix eBioscience) were also used as positive control. After washing three times with 1X TBST wash buffer, 200 µl blocking buffer were loaded into each well and incubated for 1 h at room temperature. IgG extracted from affinity method was used as primary antibody and horseradish peroxidase conjugated goat antihuman IgG antibody (Santa-Cruz) was added. Subsequently, 100 µl of the substrate (tetramethylbenzidine, eBiosciences) was loaded into each well and analyzed in ELISA reader (Multiskan Express, Thermo) at 450 nm.[19]

Tandem Mass Spectrum (MS) Analysis of Proteins (MS/MS)

For electrospray ionization-MS/MS measurement, lyophilized peptides and glycopeptides were dissolved in 25 µl purified water (MS-grade) containing 2% ACN and 0.1% FA. Peptide mixture was analyzed by nanospray MS/MS using an ultimate 3000 Nano LC System (Thermo Fisher Scientific) coupled to the LTQ Orbitrap Velos (Thermo Fisher Scientific) instrument. The sample was loaded onto an Acclaim® PepMap100 C18 column (75 µm i.d. x 15 cm length) (Thermo Fisher Scientific) equipped with a pre51 column of the same packing material (dimensions: 100 µm i.d. × 2 cm length) and separated at a flow rate of 300 nL/min using a linear gradient of 7-35% solvent B (80% ACN and 20% water with 0.08% FA) in 30 min, followed by an increase to 40% solvent B in 2 min (held for 2 min), and then to 80% B in 2 min (held for 4 min). Solvent A was water containing 0.1% FA. Protein bands were processed for mass spectrometric fingerprinting using a Reflex III instrument to determine the N-terminal end sequence of a peptide.[20]

Fourier Transform Infrared Spectroscopy (FTIR) Analysis of Proteins

Spectra were collected using a Bruker Tensor 27 FTIR spectrometer (Bruker, Germany), with ZnSe crystal cell ATR (PIKE, USA) equipped with a TE-deuterated L-alanine doped triglycine sulfate detector. Spectra were measured at room temperature, at resolution of 4/cm, 16 scans for each sample in a wavenumber range of 4000/cm to 600/cm. A background spectrum was scanned before the analysis of each sample to cancel the effect of air components in the spectrum.[21] After each measurement, the crystal was wiped off with lab soft tissue wetted by alcohol. Collected spectral data were subjected to a baseline correction (rubber band method) and vector normalization by software named OPUS 5.5. Final analysis was then accomplished by in-house software using MATLAB 2014b (MathWorks, Natick, MA, USA).

RESULTS AND DISCUSSION

SDS-PAGE Analysis of Proteins

SDS-PAGE analysis of pollens under nonreduced conditions revealed all the major pollen allergens

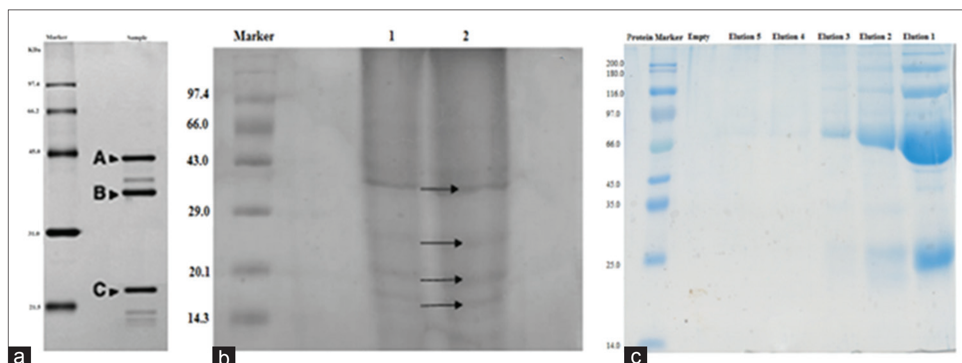

Figure 1: Sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis of grass pollens under nonreduced (a), reduced conditions (b), and immunoglobulin G (IgG) purified by affinity preparation (c) on 12% gel along with protein marker. Medium range protein ladder (97.4-14.3 kDa; Puregene) was used to determine the molecular weight of allergen proteins, represented in Figure 1a and b. High range protein ladder (200-14.3 kDa; Puregene) was used to determine the molecular weight of IgG, represented in Figure 1c

with molecular weights, 42 kDa, 38 kDa, and 23 kDa [Figure 1a] which were eluted within 10s of extraction. While under reduced conditions, studies have identified four proteins with molecular weights, 40 kDa, 23 kDa, 20 kDa, and 18 kDa [Figure 1b], respectively, as the major allergens of *Parthenium* pollen. Affinity chromatographic purification yielded Ig from serum samples with high purity, as evident from the two major bands of 75 kDa and 25 kDa [Figure 1c].

Determination of Allergen Binding Toward IgG by ELISA

Binding assay reported the increase in concentration of IgG (20 ng/ml-100 ng/ml) toward *P. hysterophorus* (50 ng/ml) pollen proteins by ELISA at an absorbance of 450 nm. As shown in Figure 2, IgG shows a good sensitive increase in concentration with maximum absorbance at 100ng/ml toward the *P. hysterophorus* allergen, which could be correlated to the retention of IgG structure for binding of antigen on its paratope region. However, there was a decrease of antibody concentration observed at 50 ng/ml concentration which might be due to the conformational changes of allergen proteins.

2D SDS-PAGE

We have analyzed 45 spots by 2D-PAGE (identified by arrows) and obtained identification for 32 of them, corresponding to four different IgG binding proteins. From these eight proteins, only four had already been identified as allergic IgG binding proteins [4]. The IgG-binding proteins [Figure 3] were heterogeneous in terms of molecular mass and isoelectric point (pI). Most of these proteins located around pI 10 and molecular mass of 16-18 kDa.

Immunoblotting

Protein sample of 20 µg was loaded per lane for electrophoretic separation. Blotting transfers of the proteins onto 0.2 µm nitrocellulose membranes were confirmed for 2 h at 125 mA in Genetix transblot system. After transfer, the membrane was stained using Ponceau staining for rapid detection [Figure 4a], and chromogenic detection was developed with TMB substrate to visualize single blue-colored band with a mass of 18 kDa [Figure 4b].

HPLC Analysis of IgG

Antibodies were further purified by reversed-phase HPLC using Schimadzu LC20AT HPLC-Phenomenex Luna 5u C18 column (250 mm × 4.6 mm) in a 10-70% ACN gradient. For this purpose, approximately, 20 µl of sample was dissolved in 2 ml 20-80% ACN and 0.1% TFA and injected onto HPLC-pump. In these conditions, the peak

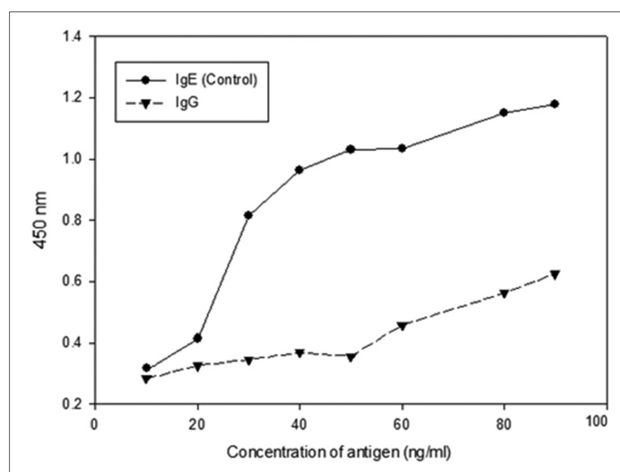

Figure 2: Increasing concentration of human immunoglobulin G activity toward antigen by ELISA

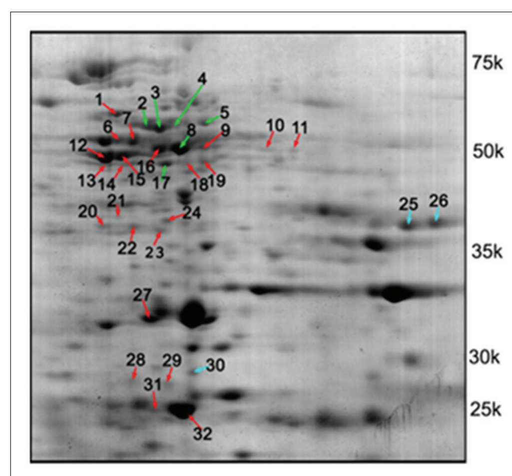

Figure 3: Proteins separated by two-dimensional PAGE showing major spots at pH 11 of molecular mass 18-20 kDa

had a broad shape with a long tail; it was possible to reduce the tailing factor by varying the concentrations of ACN and TFA combinations. As reported earlier for control IgG sample with a flow rate of 1 ml/min was retained at 22 min [Figure 5].

Tandem MS Analysis of Proteins

For the identification of glycan structures, the MS/MS spectra after CID fragmentation were carried out for manual evaluation of the allergen. First, spectra of glycopeptides were selected by searching for specific B-fragment ions typical for glycan fragmentation, (e.g., HexNAc-Hex [m/z 366.190] fragment). Based on the identified spectra, the monoisotopic precursor mass (M+H) was calculated and taken for GlycoMod searching algorithm.[22] Monoisotopic precursor masses together with UniProtKB numbers of N-linked oligosaccharides (National Center for Biotechnology Information/Blastp) was considered to find possible glycopeptides using GlycoMod search options [Figure 6]. Selected mass values were then taken to search

a protein using the GlycoMod search, which revealed the possible presence of oligos as glycans in the allergic protein.

N-terminal end sequence of peptide was found to be as EHIYAAFHLSKGD [Figure 7].

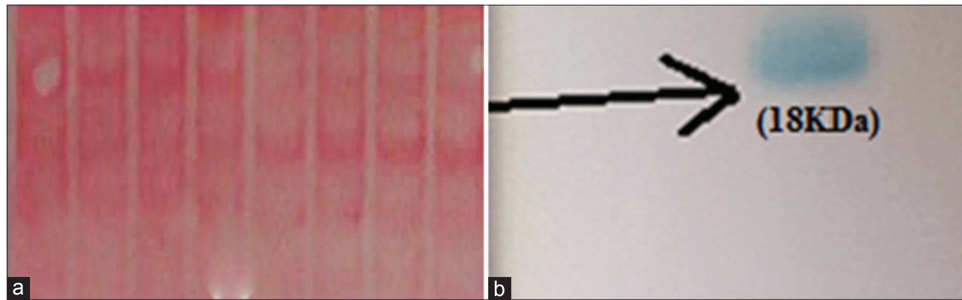

Figure 4: Immunoblotting membrane of *Parthenium hysterophorus*, stained with Ponceau reagent for rapid detection of bands transferred to the membrane (a) and arrow indicates band on the nitrocellulose membrane (b) hybridized with goat antihuman antibody with the molecular mass of 18 kDa

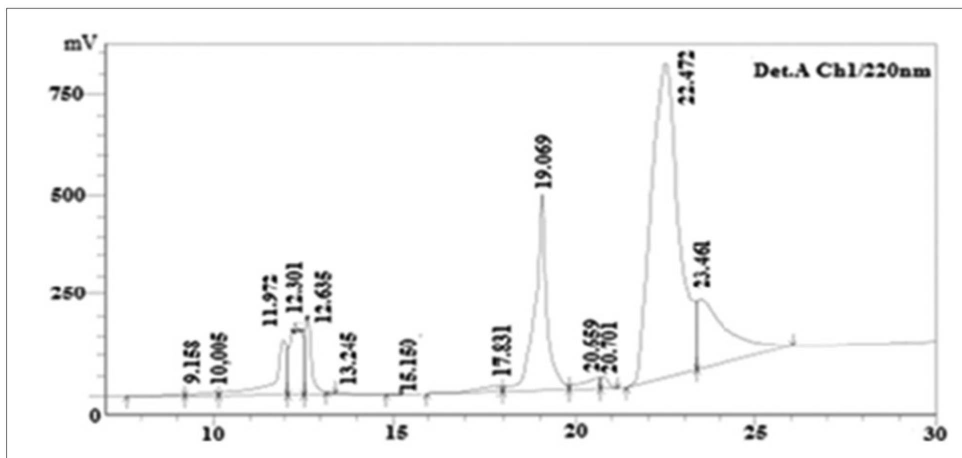

Figure 5: High-performance liquid chromatography (HPLC) analysis of immunoglobulin G (IgG). Control IgG (Merck Biosciences) was used as standards before HPLC analysis of purified Igs

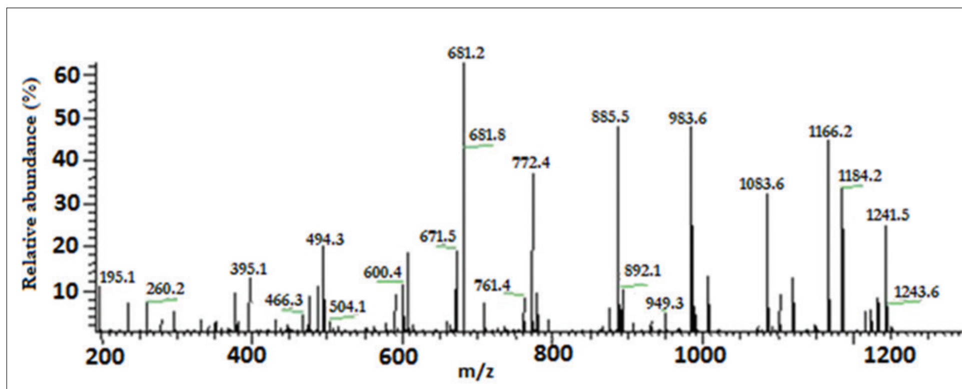

Figure 6: Mass spectrum (MS)/MS spectrum of the indicated glycopeptide located in the N-terminal domain

Peptides containing the motif 'N-X-S/T/C (X not P)':

position	#MC	peptide mass [M]	peptide	modifications
1-14	0	1616.79467	NTSEHIYAAFHLSK	

Figure 7: N-terminal end sequence of peptide determined using the GlycoMod search

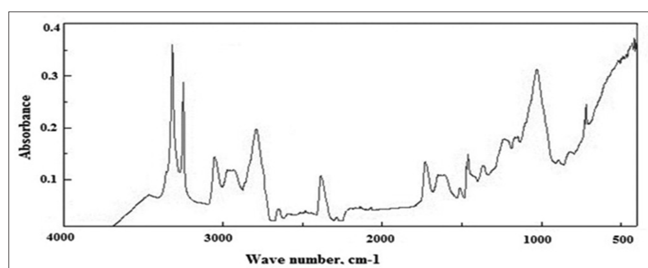

Figure 8: Fourier transform infrared spectroscopy spectra of *Parthenium hysterophorus* allergen. The absorbance spectrum shows bands corresponding to the amides and proteins

FTIR Analysis of Proteins

FT-IR spectra of *P. hysterophorus* samples were analyzed at a resolution of 4/cm at 100-150 scans. The absorbance spectrum reported bands corresponding to amide I, amide II, and amide III regions [Figure 8]. The spectra were recorded at 20° C using CaF_2 windows fitted with 6 µm tin spacers. The amide II band was observed to have overlapping component bands that represent different structural elements such as α-helices, β-sheets, turns and nonordered or irregular structures.[23] The width of the contributing component bands is usually greater than that of the separation between the maxima of adjacent peaks. As a consequence, the individual component bands could not be resolved.

CONCLUSION

P. hysterophorus is a pollen allergen source with several IgG binding components. The allergenic proteins present in the pollens of *P. hysterophorus* were characterized by SDS-PAGE, 2D-electrophoresis, and mass spectrometry biochemically and by immunoblot and IgG-ELISA immunologically. The investigation of cross-reactivity between allergens from the same protein family and different allergen sources can contribute to an improved allergenic risk management. Identification of the recombinant forms of common allergens of these pollens may lead to the search of new strategies for diagnostic and preventive purposes. As an immediate consequence, we expect allergen monitoring to serve as a predictor of human allergic symptoms better than pollen count.

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