

Delineation of the Olive Pollen Proteome and Its Allergenome Unmasks Cyclophilin as a Relevant Cross-Reactive Allergen

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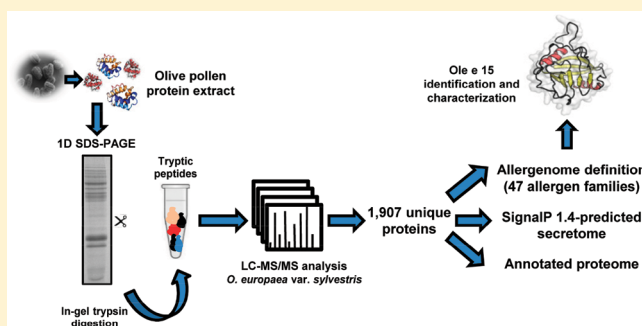
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Supporting Information

ABSTRACT: Olive pollen is a major allergenic source worldwide due to its extensive cultivation. We have combined available genomics data with a comprehensive proteomics approach to get the annotated olive tree (*Olea europaea* L.) pollen proteome and define its complex allergenome. A total of 1907 proteins were identified by LC–MS/MS using predicted protein sequences from its genome. Most proteins (60%) were predicted to possess catalytic activity and be involved in metabolic processes. In total, 203 proteins belonging to 47 allergen families were found in olive pollen. A peptidyl–prolyl *cis*–*trans* isomerase, cyclophilin, produced in *Escherichia coli*, was found as a new olive pollen allergen (Ole e 15). Most Ole e 15-sensitized patients were children (63%) and showed strong IgE recognition to the allergen. Ole e 15 shared high sequence identity with other plant, animal, and fungal cyclophilins and presented high IgE cross-reactivity with pollen, plant food, and animal extracts.

KEYWORDS: olive pollen proteome, allergenome, in-depth proteomics, allergen, cyclophilin, cross-reactivity



INTRODUCTION

The pollen grain has aroused much interest not only for its role as the male gametophyte in reproduction¹ but also for its major role as an allergen source. Proteomics studies have shown that proteins acting in pollen formation and germination are mainly involved in energy production, defense, and protein synthesis and processing.^{2–4} Moreover, as remarked above, some pollen proteins are capable of triggering airway inflammation and antigenic responses, which, in some individuals, cause allergy.^{5–8} For this reason, the proteomics analysis of pollen can be used for allergen identification, as has been done for several allergenic sources.^{9–13} However, there is limited genomics information on tree species with allergenic pollen, reducing the potential number of these studies.

Recently, the complete genome of the wild olive (*Olea europaea* subsp. *europaea* var. *sylvestris*; also denominated as oleaster), a closely related variant of cultivated olive (*O. europaea* subsp. *europaea* var. *europaea*), has been reported.¹⁴ Cultivated olive belongs to the Oleaceae family (order

Lamiales) and has been traditionally cultivated in the Mediterranean Basin, being the main crop in some areas of Spain, Italy, and Greece.¹⁵ In addition, olive trees are extensively cultivated worldwide for olive oil and olive fruit production. Several regions of these countries reach extremely high olive pollen counts in the pollination season, and olive pollen has become a major allergenic health problem.¹⁶ Until now, 13 olive pollen allergens, Ole e 1 to Ole e 12 and Ole e 14, have been identified.^{17–19} However, some allergens have remained unidentified due to technical restrictions and limited genomics and proteomics data. Moreover, very little is known about the other proteins present in this pollen, which although not allergenic, may influence the immune response of patients by promoting inflammation.^{20–22}

In this work, we have performed a comprehensive proteomics analysis of cultivated olive pollen, taking advantage

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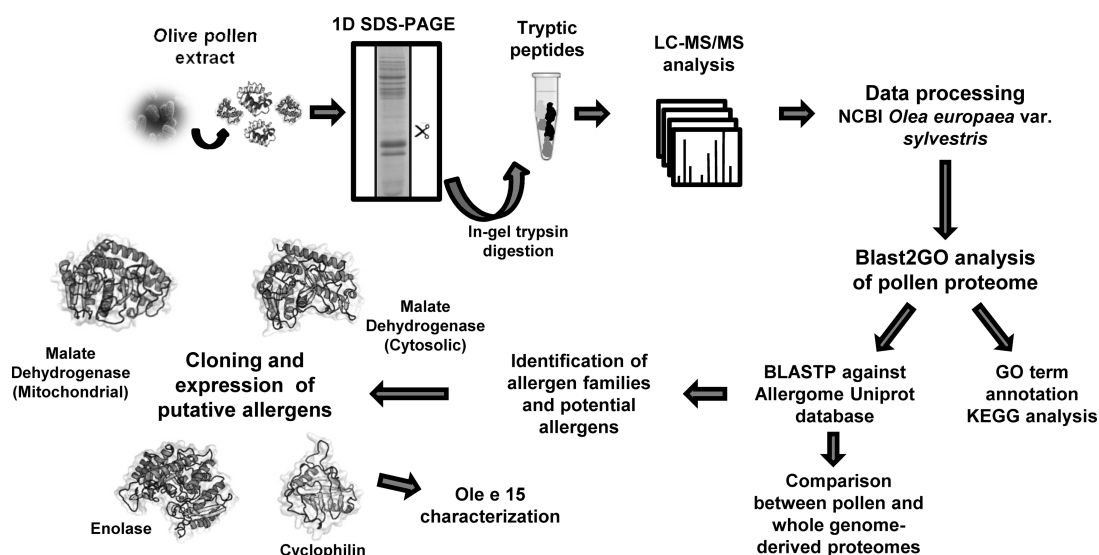


Figure 1. Workflow of the study to delineate the olive pollen proteome and its allergenome and to describe Ole e 15 as a new relevant allergen implicated in cross-reactivity. 3D models of the proteins were created using 1SEV.1.A, 5.NUE.1.B, 1XSX.1.A, and 2MC9 PDB structures for malate dehydrogenase (mitochondrial), malate dehydrogenase (cytosolic), enolase, and cyclophilin, respectively.

of the wild olive genomics data. By an in-depth bottom-up proteomics approach, the olive pollen proteome has been identified in-depth using a Q-exactive mass spectrometer and functionally catalogued according to Gene Ontology (GO) terms. We next extended our analysis to the definition of the olive pollen allergenome, identifying its allergen families by searching the proteomics data with the Allergome, World Health Organization and International Union of Immunological Societies (WHO/IUIS), and AllFam (the database of allergen families) databases.^{23–25} Among them, we finally focused the study on the verification of the allergenicity of four proteins not previously studied in olive pollen because of their potential involvement in cross-reactivity with other allergenic sources. We found cyclophilin, a peptidyl-prolyl *cis*–*trans* isomerase, as a new relevant olive pollen allergen. Enzyme-linked immunosorbent assay (ELISA), Western blot (WB), and immunoglobulin E (IgE) inhibition assays revealed its significance, high potency, and wide range of cross-reactivity with pollen, plant-derived food, dust mites, and its human counterparts.

METHODS

Protein Extracts

Commercial olive pollen from Allergon (batch no. 032509801, Ängelholm, Sweden) collected in California from a controlled set of cultivated olive trees belonging to different cultivars was used for protein extract preparation for proteomics. Three parallel protein extractions were performed and used later as independent biological replicates. In brief, for each biological replicate, 300 mg of olive pollen was separately resuspended in 6 mL of extraction buffer (100 mM Tris-HCl pH 7.3, 150 mM NaCl, 2 mM EDTA, 10% glycerol, 1% Triton X-100, and 1 mM phenylmethanesulfonyl fluoride (PMSF) with 1× Complete Protease Inhibitor Cocktail (Roche, Basel, Switzerland)). After homogenization, the resultant emulsion was subjected to mild stirring for 90 min at room temperature. Then, it was transferred to 2 mL tubes and centrifuged at 12 000g for 20 min at 4 °C. The insoluble material was resuspended again in 1.5 mL of extraction buffer and stirred for

1 h. After centrifugation, both supernatants were pooled together, and the sample was analyzed by SDS-PAGE.

Pollen protein extracts and plant-derived food protein extracts were prepared for the other assays according to established protocols,²⁶ and their concentrations were measured using the Lowry protein assay.

SDS-PAGE, Trypsin Digestion, and LC–MS/MS Analysis

First, 40 µg of each extract replicate were separated by 15% SDS-PAGE and stained with Coomassie Brilliant Blue G 250 (Merck, Darmstadt, Germany). Then, whole lanes were cut into four (replicate 1) or six slices (replicates 2 and 3) prior to in-gel trypsin digestion.^{27,28} Then, excised bands were cut into small pieces and separately destained with 50 mM ammonium bicarbonate (ABC)/50% acetonitrile (ACN), dehydrated with ACN, and dried. Samples were then reduced with 10 mM dithiothreitol (DTT) in 25 mM ABC and alkylated with iodoacetamide to a final concentration of 50 mM. Then, gel pieces were dried, rehydrated with 12.5 ng/mL porcine trypsin (Thermo Fisher Scientific, Waltham, MA) in 50 mM ABC, and incubated overnight at 37 °C. Peptides were extracted using 100% ACN and 0.5% trifluoroacetic acid, purified using a Zip Tip with 0.6 µL of C18 resin (Millipore, Sigma-Aldrich Química SL, Madrid, Spain), and dried. Finally, samples were reconstituted in 5 µL of 0.1% formic acid/2% ACN before the analysis by nLC–MS/MS in the Proteomics and Genomics Facility of the Centro de Investigaciones Biológicas (CIB-CSIC, Madrid, Spain), a member of ProteoRed-ISCI network.

All peptide separations were carried out on an Easy-nLC 1000 nanosystem (Thermo Fisher Scientific). For each analysis, the sample was loaded into an Acclaim PepMap 100 precolumn (Thermo Fisher Scientific) and eluted in an RSLC PepMap C18 column (15 cm long, 50 µm inner diameter and 2 µm particle size) (Thermo Fisher Scientific). The mobile phase flow rate was 300 nL/min using a gradient of 0.1% formic acid in water (solvent A) and 0.1% formic acid and 100% ACN (solvent B). The gradient profile was set as follows: 5–35% solvent B for 45 min, 35–100% solvent B for 5

min, and 100% solvent B for 10 min. Four microliters of each sample was injected.

MS analysis was performed using a Q-Exactive mass spectrometer (Thermo Fisher Scientific). For ionization, 2000 V of liquid junction voltage and a 270 °C capillary temperature were used. The full-scan method employed a m/z 400–1500 mass selection, an Orbitrap resolution of 70 000 (at m/z 200), a target automatic gain control (AGC) value of 3e6, and maximum injection times of 100 ms. After the survey scan, the 10 most intense precursor ions were selected for MS/MS fragmentation. Fragmentation was performed with normalized collision energy of 27, and MS/MS scans were acquired with a starting mass of m/z 100. The AGC target was 2e5, the resolution was 17 500 (at m/z 200), the intensity threshold was 8e3, the isolation window was 2 m/z units, and the maximum IT was 100 ms. Charge-state screening was enabled to reject unassigned, singly charged, and ≥ 7 protonated ions. A dynamic exclusion time of 20 s was used to discriminate against previously selected ions.

Data Processing

MS data were analyzed with Proteome Discoverer (version 1.4.1.14, Thermo Fisher Scientific) using standardized workflows. Mass spectra raw files were searched against the NCBI *Olea europaea* var. *sylvestris* predicted protein database (taxid 158386, version 11/2017, 49 613 RefSeq protein sequence entries) using the SEQUEST search engine. Precursor and fragment mass tolerance were set to 10 ppm and 0.02 Da, respectively, allowing two missed cleavages, carbamidomethylation of cysteines as a fixed modification, and methionine oxidation and threonine, tyrosine, and serine phosphorylation as a variable modification. Identified peptides were filtered using the Percolator algorithm with a q -value threshold of 0.01 (High Confidence Filter settings, FDR < 1%).²⁹ The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository, with the data set identifiers PXD012334 (Replicate 1), PXD012279 (Replicate 2), and PXD012280 (Replicate 3).

Experimental and Statistical Design

The experimental and statistical designs for each experiment of the study are described in each subsection and depicted in Figure 1 and Figure S1. Only proteins containing at least two isoform-specific peptides in one of the replicates or with one high-confidence isoform-specific peptide but detected in the three replicates were considered in the study. To identify proteins belonging to allergen families, the full sequences of the identified proteins after LC–MS/MS were retrieved using NCBI Batch Entrez. Then, proteins were locally blasted (E -value threshold 1.0×10^{-15} and one Blast hit) against all entries of the Allergome database (4331 entries; UniProt release 10/2018) using the Blast2GO 5 PRO software (5.2.4 version).³⁰ Sequence similarity was calculated by Blast2GO, and sequence identity was calculated using Clustal Omega.³¹ Only those proteins meeting the following criteria were considered as potential allergens: matching with an allergen accepted by the WHO/IUIS Allergen Nomenclature Subcommittee²⁵ with at least 50% similarity; matching directly with proteins belonging to the same allergen families as proteins meeting the previous criteria, with at least 80% similarity; matching with proteins already identified as potential allergens in the Oleaceae family.

Gene Ontology (GO) Annotation and Prediction of Secreted Proteins

To perform the functional GO annotation and KEGG analysis of each protein, we used the Blast2GO 5 PRO software (5.2.4 version). First, we created a database with the curated NCBI RefSeq predicted proteomes from rice (*Oriza sativa*, taxid: 4530), *Arabidopsis thaliana* (taxid: 3702), latex (*Hevea brasiliensis*, taxid: 3981), barrelclover (*Medicago truncatula*, taxid: 3880), black cottonwood (*Populus trichocarpa*, taxid: 3694), peach (*Prunus persica*, taxid: 3760), tomato (*Solanum lycopersicum*, taxid: 4081), potato (*Solanum tuberosum*, taxid: 4113), and common grape vine (*Vitis vinifera*, taxid: 3603). GO annotations for these curated species are deposited in Ensembl Plants³² and are accessible through Blast2GO by BioMart.³³ Then, olive-pollen-identified proteins were locally blasted against this database (maximum number of BLAST hits: 1; minimal E value: 1.0×10^{-50} ; BLAST descriptor annotator activated; HPS length cut off: 50; rest of parameters: default). GO mapping (GOA version 09.2018, default settings) and annotation (taxonomy filter: taxa: 33090, Viridiplantae) were performed. Additionally, the InterProScan (IPS, default settings) analysis was run,³⁴ and the annotated IPS GOs were merged with previous GOs. Kyoto Encyclopedia of Genes and Genomes (KEGG) Enzyme codes were also annotated. Further information about molecular pathways was obtained by KEGG analysis.³⁵ For Fisher's exact test enrichment analysis of molecular function and biological process GOs, the obtained olive pollen annotation was tested against the whole *O. europaea* var. *sylvestris* predicted proteome and annotated using the same parameters as described above for pollen proteins. Only GO terms with p value $< 1.0 \times 10^{-10}$ were considered as enriched.

Secreted proteins were predicted using the SignalP 4.1 (organism group: eukaryotes; D -cutoff value 0.45 for Signal P-noTM and TM networks).³⁶

Analytical Procedures

1D SDS-PAGE (15 or 17% gels) was stained with Coomassie Blue R-250 (Sigma-Aldrich, St. Louis, MO) or transferred onto nitrocellulose membranes. Molecular mass calculations were performed using unstained protein biomarkers SM0431 (Fermentas, Waltham, MA) or prestained protein molecular weight markers (Precision Plus Protein All Blue and Prestained Protein Standards low range from Bio-Rad, Hercules, CA).

For two-dimensional gel electrophoresis (2DE), isoelectric focusing of 80 μ g of olive pollen extract was achieved under reducing conditions with 3 mM tributylphosphine in a PROTEAN IEF cell (Bio-Rad) using pH 3–10, 7 cm length linear ReadyStrip IPG gels (Bio-Rad). After isoelectrofocusing, proteins were separated by 17% SDS-PAGE under reducing conditions in the presence of 50 mM DTT and 3.7% iodoacetamide and transferred onto nitrocellulose membranes (Amersham Biosciences, Little Chalfont, U.K.) according to the Towbin method for WB.

The protein concentration of the purified proteins was calculated by measuring the absorbance at 280 nm using a DU-7 spectrometer (Beckman, Barcelona, Spain) after the theoretical extinction coefficient calculation with the ProtParam tool from ExPASy. The rOle e 15 concentration was calculated using the experimental extinction coefficient ($E^{0.1\%}$) of 0.616, obtained after amino acid analysis of 5 and 10 μ g of purified protein in duplicate with a BioChrom 30 amino acid analyzer (Harvard Bioscience, Holliston, MA).

Patients' Sera and Antibodies

A total of 146 sera of patients allergic to olive pollen were used to carry out the study. All samples were handled anonymously according to the ethical and legal guidelines of the Complutense University of Madrid (CEI PI 49) and Reina Sofia University Hospital (ref. 3033). Written informed consent was obtained from all patients. Sera from olive-pollen-allergic patients according to their positive olive pollen skin prick test (SPT) and positive profile by ImmunoCAP 250 (Thermo Fisher Scientific, Uppsala, Sweden) were obtained from two different Spanish populations (Córdoba and Madrid, Spain) according to standard operating procedures. Values >0.35 kU/L by ImmunoCAP were considered positive. SPTs were performed according to standard procedures. A wheal diameter <3 mm was considered negative. All patients included in this study reported an immediate IgE-mediated allergic reaction and a positive SPT with olive pollen. As a summary of the pollen sensitization of each population: sera from Madrid ($n = 42$) were all cosensitized to *Lolium perenne*, whereas sera from Córdoba ($n = 104$) were sensitized almost exclusively to olive pollen (Table S1). All patients presented asthma or rhinoconjunctivitis.

Rabbit polyclonal antiserum against rOle e 15 was obtained by injections of the protein (100 μ g) in complete Freund's adjuvant, accomplishing the ethics guidelines of the Fundación Jiménez Díaz Hospital (Madrid, Spain). Horseradish-peroxidase-labeled goat polyclonal antibody against rabbit IgG was purchased from Bio-Rad. Horseradish-peroxidase-labeled mouse antihuman IgE was purchased from Southern Biotech (Birmingham, AL).

Circular Dichroism Spectroscopic Analyses, Structural 3D Modeling, and Alignments

The circular dichroism (CD) spectrum of rOle e 15 (0.2 μ g/ μ L) in 20 mM phosphate buffer, pH 7.0 was recorded in the far-UV (190–260 nm) on a JASCO J-715 spectropolarimeter (Japan Spectroscopic, Tokyo, Japan) using a 0.1 cm optical-path quartz cuvette and at 20 °C, 85 °C, and after cooling to 20 °C. The deconvolution of the obtained spectra was performed using the CDNN software. Thermal unfolding was followed measuring the ellipticity at 226 nm heating (20 to 85 °C) or cooling (85 to 20 °C) at 0.5 °C/min with a computer-controlled water-bath. Far-UV spectra were baseline-subtracted and represented as the mean residue molecular ellipticity.

Sequence identity and similarity of the Ole e 15 amino acid sequence against other plant, animal, and fungal cyclophilins were calculated using DIALIGN (Bielefeld Bioinformatics Service, Universität Bielefeld, Germany). The results were visualized using the GeneDoc program. Known cyclophilin structures were downloaded from PDB. Structural models in PDB format were generated using the ExPASy Swiss-Model tool with human (PDB: 1OCA) and Cat r 1 (PDB: 2MC9) as templates. Final 3D-structure models were visualized with PyMOL (Schrödinger, New York, NY). The electrostatic surface potential of rOle e 15 was calculated by the Adaptive Poisson–Boltzmann Solver (APBS) program³⁷ and visualized with PyMOL.

Immunological Characterization

Indirect ELISA was performed in 96-well plates (Costar, Corning, NY) coated with 0.1 μ g of recombinant protein per well, according to previously optimized protocols,^{18,38} using individual serum samples diluted 1:10.

WB analysis of human IgE bound to alternatively 0.5 μ g rOle e 15 or 40 μ g of olive pollen extract immobilized onto nitrocellulose membranes was performed incubating with individual human sera (1:10) and horseradish-peroxidase-labeled mouse antihuman IgE Fc (1:1000). The binding of specific polyclonal antisera against rOle e 15 was detected by goat antirabbit IgG horseradish-peroxidase-labeled antibody (1:3000). The chemiluminescence signal was developed using ECL-Western blotting reagent (Amersham Bioscience) or WesternBright QUANTUM (Advansta, San Jose, CA) reagents.

For the IgE inhibition experiments with indicated protein extracts, a pool of sera ($n = 5$ for WB; $n = 6$ for ELISA) was preincubated with 5 mg/mL for WB or alternatively 0.25 and 2.5 mg/mL for ELISA of the inhibiting extracts for 2 h at room temperature with shaking. The following steps of the ELISA or WB experiments were performed as described above, and the results are shown as the percentage of inhibition.^{18,38} For the IgE inhibition experiments using purified proteins, serum was preincubated with 10 μ g of rOle e 15, nOle e 1, or both. IgE reactivity was visualized as previously described.^{18,35} Inhibition values were calculated as a percentage in comparison with the noninhibited control using the formula $[(1 - OD_{492nm} \text{ with inhibitor} / OD_{492nm} \text{ without inhibitor}) * 100]$ by ELISA and as the signal lost in comparison with a noninhibited control (no inhibition) by WB after densitometry of the bands using the Quantity One 1-D analysis software (Bio-Rad Laboratories, Madrid, Spain).

Cloning of the Putative Allergens from *O. europaea* Pollen, Expression, and Purification

Total RNA purification from pollen was performed using the TRIzol and RNeasy Plant MiniKit (Qiagen, Hilden, Germany). The total RNA quality and concentration were analyzed using a 2100 Bioanalyzer (Agilent Genomics). cDNA amplification was performed using the SMARTer RACE 5'/3' kit (Clontech, TaKaRa Bio Company, Mountain View, CA) or the SuperScript III First-Strand Synthesis System (Thermo Fisher Scientific). Different degenerate and nondegenerate oligonucleotides (Table S2) were synthesized based on the available proteomics and genomics data. Polymerase chain reaction (PCR) products were cloned into the pCR2.1 plasmid using the TOPO TA cloning kit (Invitrogen, Groningen, The Netherlands) and sequenced. Then, specific overlapping oligonucleotides (Table S2) were designed to subclone the cDNAs encoding the complete proteins into the pET28a plasmid (Novagen, Billerica, MA) as N-terminal His₆-tagged proteins using, alternatively, the NEBuilder HiFi DNA assembly mix (New England Biolabs, Ipswich, MA) or the InFusion HD cloning kit (Clontech), following the manufacturer instructions.

The pET28a/cDNA constructs were used to transform BL21 (DE3) *E. coli* cells. Ten mL of 30 μ g/mL kanamycin/LB cultures containing the transformed cells were grown overnight and then diluted ten times and grown until OD_{600nm} reached 0.7. Finally, the protein production was induced with 0.4 mM isopropyl thio- β -D-thiogalactoside (IPTG) and grown at 30 °C at 230 rpm for 16 h. Then, the cultures were centrifuged at 6000g for 20 min at 4 °C. Proteins were purified by gravity-flow chromatography from the soluble fraction of cell lysates using Ni-NTA agarose (Qiagen), following the manufacturer's instructions with minor modifications. In brief, cell pellets corresponding to 250 mL of *E. coli* culture were resuspended in 10 mL of lysis buffer (300 mM NaCl, 1 mM PMSF, 50 mM

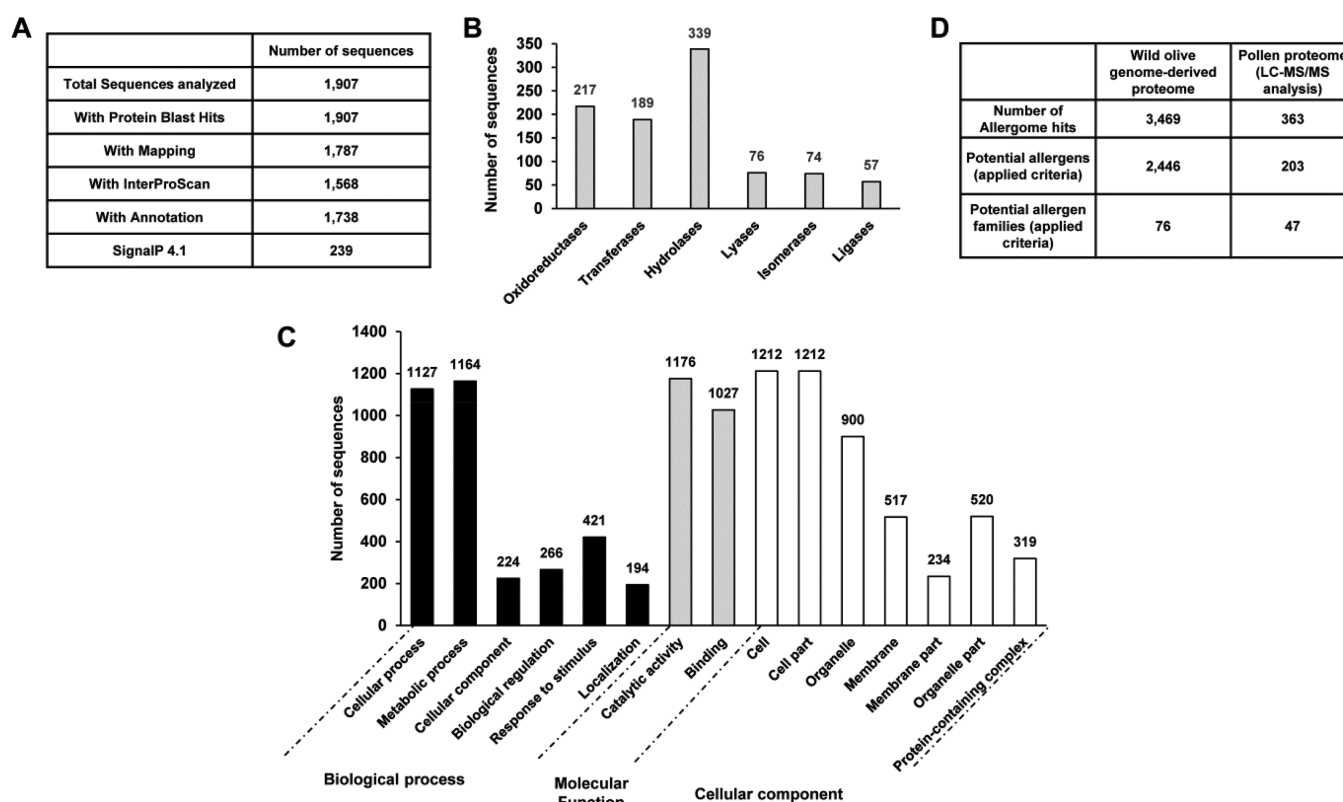


Figure 2. Classification of the olive pollen proteome according to the annotated GO terms after Blast2GO analysis and identification of potential allergens. (A) Summary table with the general results after Blast2GO and SignalP 4.1 bioinformatic analysis. (B) Biological process, molecular function, and cellular component GO term distribution at level 2 of complexity. (C) Number of proteins of each enzyme class after GO-Enzyme Code mapping. (D) Table summarizing the main results after protein BLAST against the Allergome database and application of criteria for the identification of potential allergens and allergen families.

phosphate buffer pH 8.0), distributed in 1 mL aliquots, and disrupted by five cycles of subsequent freezing in liquid N₂ for 40 s and thawing at 42 °C in a water bath for 5 min. Then, soluble fractions obtained by centrifugation at 6000g for 20 min at 4 °C were incubated for 16 h at 4 °C in a 15 mL conical centrifuge tube with 500 μ L of Ni-NTA (CliniSciences, Nanterre, France) agarose previously preincubated three times with 5 mL of lysis buffer. Then, Ni-NTA agarose was washed five times for 10 min at 4 °C with 5 mL of wash buffer (10 mM imidazole, 300 mM NaCl, 50 mM phosphate buffer pH 8.0); each time the resin was recovered by centrifugation at 4000g for 10 min at 4 °C. Proteins were then eluted with 5 mL of elution buffer (500 mM imidazole, 300 mM NaCl, 50 mM phosphate buffer pH 8.0) for 20 min at 4 °C on rotation. To avoid the presence of traces of Ni-NTA agarose in the eluted fractions, a final centrifugation step was performed at 12 000g for 5 min at 4 °C. Finally, purified proteins were desalted onto PD10 desalting columns (GE Healthcare, Chicago, IL) with 20 mM ABC, quantified, aliquoted, lyophilized, and stored at –20 °C until use.

RESULTS

Definition of the Olive Pollen Proteome by Proteomics and Bioinformatics

In the present study, we have used an in-depth bottom-up proteomics approach to analyze the proteomics profile of olive pollen, to classify its allergen families and define its allergenome, and to characterize new olive pollen allergen candidates (Figure 1).

First, three olive pollen protein extracts replicates were fractionated by SDS-PAGE, and protein bands were in-gel trypsin digested. Then, the extracted peptides were analyzed by LC-MS/MS with a Q-exactive mass spectrometer. Next, we investigated the quality of the MS results by calculating the mean and SD of the mass error in ppm in the three replicates. For replicate 1, the mean $\pm$ SD mass error were 0.66 and 1.84, for replicate 2, they were 2.39 and 1.64, and for replicate 3, they were 2.41 and 1.68. Because the data showed good quality, we proceeded to subsequent analyses.

From the three biological replicates, a total of 1907 different proteins were identified (Table S3 and Figure S1). Protein sequences identified from olive pollen were functionally mapped and annotated with the corresponding GO terms using the Blast2GO software after local protein BLAST with NCBI RefSeq sequences from plant species with curated GO annotations from Ensembl Plant (see the Methods). A total of 1738 pollen proteins were annotated with GO terms after combining GO mapping and InterProScan data (Figure 2A and Table S4).

Cellular component terms revealed that identified olive pollen proteins were widely distributed among the cell, with more than 500 proteins associated with membranes. Metabolic processes were the most abundant in biological process terms (Figure 2B and Figure S2). Regarding molecular function, cyclic compound, ion and protein binding activities, and hydrolase activity were highly represented (Figure S2). It is noteworthy that 1176 out of the 1907 identified proteins displayed catalytic activity (61.7%), with hydrolases, oxidore-

eductases, and transferases being the most abundant enzyme classes found (Figure 2C and Table S4). KEGG pathway analysis showed that 7 out of the 10 most enriched pathways were related to metabolism (Figure S3a). To analyze the enrichment of certain GO terms in pollen, a Fisher's exact test was performed comparing the annotated GO terms of the pollen proteome with those of the genome-derived proteome (Figure S3b and Table S4). GO terms related to salt stress, salt absorption, and amino acid and sugar metabolism were over-represented in pollen. Moreover, 239 (12.5%) of the identified pollen proteins were predicted, as secreted by SignalP 4.1 (Table S5).

Olive Pollen Allergenome Delineation Reveals the Presence of 47 Allergen Families

Next, the delineation of the olive pollen allergenome was investigated to identify putative allergens in this allergenic source with high clinical relevance.¹⁷ First, the whole wild olive genome predicted proteome and the olive pollen identified proteome were locally blasted against the Allergome database. A total of 3469 and 363 hits were retrieved, respectively. Then, a set of criteria (see the Methods) were manually applied to filter the results and identify potential allergens. Of the total of 2446 potential allergens belonging to 76 different allergen families in the wild olive predicted proteome, 203 proteins belonging to 47 allergen families were present in pollen (Figure 2D, Table 1, and Tables S6 and S7). Among them, 10 families previously reported as actual olive pollen allergens were observed.¹⁷ Moreover, 60 of these proteins (29.6%) were predicted to be secreted by SignalP 4.1 (Table S5), which is one of the most common features of allergenic proteins.

One of the Four Selected Allergen Candidates, Cyclophilin from Olive Pollen, Shows Significant IgE Reactivity

After proteomics analysis, 4 potential allergens among the 47 allergen families identified from olive pollen (cyclophilin, enolase, and cytosolic and mitochondrial malate dehydrogenases (MDHs)) were selected for validation to assess their IgE reactivity. Three different cDNA clones from cyclophilin and one cDNA clone for the rest of the proteins were obtained (Table S8). All proteins shared >95% sequence identity with predicted proteins in the *Olea europaea* var. *sylvestris* database.

We then proceeded to express and purify the potential allergens to analyze their IgE seroreactivity. The predicted mature forms of the proteins were expressed in *E. coli* as His₆-tagged N-terminal proteins. A purity of >95% for all proteins was achieved (Figure 3A). Proteins were subsequently analyzed by ELISA using sera from olive-pollen-allergic patients from two populations from Spain, Madrid and Córdoba, and five nonatopic individuals.

Only olive pollen cyclophilin showed IgE recognition and thus was named Ole e 15 according to WHO/IUIS (Figure 3B). No significant differences in the recognition frequency were observed between both patients' populations, with prevalence values of 14.3 and 12.5% in Madrid and Córdoba, respectively (Figure 3C).

Most of the allergic patients showed high IgE levels against the allergen, especially in the population from Córdoba. Furthermore, we observed a higher number of pediatric patients than adults among the Ole-e-15-sensitized patients. In total, 8 out of the 13 allergic patients detected in Córdoba (61.5%) and 4 out of the 6 allergic patients detected in Madrid (66.7%) were pediatric (Figure 3C).

Recombinant Ole e 15 Shows Typical Folding, Shares High Sequence Identity with Other Plant, Animal, and Fungal Cyclophilins, and Is Involved in Cross-Reactivity

Next, we proceeded to further physicochemically and immunologically characterize this new olive pollen allergen. To this end, we initially assessed by mass spectrometry and CD its correct molecular mass and folding, respectively. First, an experimental molecular mass of 19 408 Da was observed for His₆-tagged rOle e 15, in agreement with the expected theoretical molecular mass without the initial Met (19 391 Da), which is processed when proteins are produced in bacteria. Far-UV CD spectra and percentages of secondary structure of rOle e 15 were in agreement with those described for other cyclophilins. In addition, thermal denaturation experiments for rOle e 15 revealed a T_m of 47 °C, and it was unable to recover its structure cooling afterward (Figure S5).

Ole e 15 amino acid sequence alignment with other plant, animal, and fungal allergenic cyclophilins showed a high degree of conservation, with identities of 94, 68, and 65% with Sola l 5, Der f 29, and Mala s 6, respectively (Figure S6). Molecular modeling revealed a predicted structure with typical plant cyclophilin folding (Figure 4A–C and Figure S6), consisting of a β -barrel of seven antiparallel strands, two surrounding α -helices, and a characteristic stretch of seven amino acids called the divergent loop, which is mainly present in plants.⁴⁰ Moreover, the predicted amino acids defining the active surface in the cyclophilin family were present (Figure 4A).⁴¹ Its theoretical surface electrostatic potential was mainly basic but showed a neutral potential in the putative active surface region (Figure 4B). In addition, other cyclophilins were modeled and compared with Ole e 15 (Figure 4C); an almost identical folding was shown among them, except for the divergent loop.

All of these data suggested a potential implication of olive pollen cyclophilin in cross-reactivity, as has been shown for other plant and fungal cyclophilins.^{40,42,43} To address this question, we performed IgE inhibition assays with a pool of sera from olive-pollen-allergic patients using pollen, plant-derived food, dust mite, and human lung protein extracts as inhibitors (Figure 4D,E).

Remarkably, IgE inhibition was observed with all pollen and plant-derived food protein extracts from related and unrelated species, together with dust mite and human lung or cell protein extracts, by either ELISA or WB. The highest inhibition was achieved with extracts from different types of tree pollens from the Oleaceae family and birch. Regarding plant-derived food extracts, an IgE inhibition >60% was observed for all extracts analyzed by ELISA, whereas by WB, the IgE inhibition was lower for nut extracts (up to 65% for pistachio (*P. vera*)) than for fruit and seed extracts (up to 98% for sesame (*S. indicum*) or pumpkin (*C. maxima*)). Interestingly, 40 and 80% IgE inhibition by ELISA and 55 and 96% by WB were also achieved by using human dust mite (*D. pteronyssinus*) and human (*H. sapiens*) lung protein extracts, respectively.

Identification of Natural Ole e 15 Is Hampered by Ole e 1, the Main Allergen in the Olive Pollen Extract

Finally, we assessed the behavior of natural Ole e 15 (nOle e 15) in the olive pollen protein extract to determine why this relevant allergen implicated in pollen–pollen, pollen–plant food, or pollen–dust mite cross-reactivity has remained elusive in previous investigations. The most abundant protein in olive pollen extract is the major allergen Ole e 1, which, after SDS-

Table 1. Olive Pollen Allergene Definition by In-Depth Proteomics and Bioinformatics Using the Allergome Database^a

accession no. ^b	protein name	homologous allergen in the IUIS database	UniProt code	organism	amino acid similarity/identity (%)	E value	allergen family name	Allfam code	route of sensitization	total potential olive pollen allergens identified in the family ^c
XP_022892128.1	major pollen allergen. Ole e 1-like	Ole e 1	P19963	<i>O. europaea</i> (cultivated olive)	99/95	7.40×10^{-107}	Ole e 1-like	AF087	inhalation	17
XP_022882512.1	profilin-3	Ole e 2	A4GDU2	<i>O. europaea</i> (cultivated olive)	100/98	0	profilin	AF051	inhalation	4
XP_022850789.1	polcalcin	Ole e 3	O81092	<i>O. europaea</i> (cultivated olive)	100/98	0	EF-hand	AF007	inhalation	5
XP_022849259.1	superoxide dismutase [Cu–Zn] 2	Ole e 5	Q8L5E0	<i>O. europaea</i> (cultivated olive)	100/100	0	Cu/Zn superoxide dismutase	AF020	inhalation	2
XP_022896469.1	nonspecific lipid transfer protein	Ole e 7	(18) ^d	<i>O. europaea</i> (cultivated olive)	81/74	–	prolamin	AF050	inhalation	3
XP_022899478.1	glucan endo-1,3- β -D-glucosidase	Ole e 9	D8VPP5	<i>O. europaea</i> (cultivated olive)	100/99	0	β -1,3-glucanase and X8 domain	AF063	inhalation	7
XP_022888359.1	pectinesterase 1	Ole e 11	A4GDU2	<i>O. europaea</i> (cultivated olive)	99/99	0	pectin methylesterase	AF026	inhalation	9
XP_022894573.1	isoflavone reductase-like protein	Ole e 12	E1U332	<i>O. europaea</i> (cultivated olive)	100/99	0	isoflavone reductase	AF123	inhalation	5
XP_022859834.1	protein P21-like	Act d 2	L7TRX2	<i>A. deliciosa</i> (kiwi fruit)	74/57	1.69×10^{-97}	thaumatin-like protein (Ole e 13)	AF060	ingestion	1
XP_022872535.1	exopolysaccharuronase-like	Pla a 2	Q6H9K0	<i>P. xacerefolia</i> (London planetree)	73/51	2.15×10^{-142}	polysaccharuronase (Ole e 14)	AF057	inhalation	4
XP_022846733.1	enolase 2	Hev b 9	Q9LE19	<i>H. brasiliensis</i> (latex)	96/89	0	enolase	AF031	contact	4
XP_022888607.1	glutathione S-transferase L3-like	Bet v 8	A0A096XCA4	<i>B. verrucosa</i> (birch)	82/69	3.76×10^{-121}	glutathione S-transferase	AF010	inhalation	1
XP_022880502.1	cysteine proteinase inhibitor 1-like	Act d 4	Q6TPK4	<i>A. deliciosa</i> (kiwi fruit)	77/51	9.97×10^{-34}	cystatin	AF005	ingestion	1
XP_022876210.1	probable cysteine protease RD19B	Act d 1	ASHJ3	<i>A. deliciosa</i> (kiwi fruit)	91/80	0	papain-like cysteine protease	AF030	ingestion	9
XP_022857847.1	5-methyltetrahydropteroyltyrosylglutamate-homocysteine methyltransferase	Sal k 3	CIKEU0	<i>S. kali</i> (Russian thistle)	95/89	0	cobalamin-independent methionine synthase	AF152	inhalation	7
XP_022854141.1	probable pectate lyase P59	Art v 6	A0PJ16	<i>A. vulgaris</i> (mugwort)	65/49	1.63×10^{-40}	pectate lyase	AF073	inhalation	3
XP_022867183.1	superoxide dismutase [Mn], mitochondrial-like isoform X2	Hev b 10	P35017	<i>H. brasiliensis</i> (latex)	90/83	7.50×10^{-145}	Fe/Mn superoxide dismutase	AF019	contact	2
XP_022880113.1	NAD(P)H dehydrogenase (quinone) FQR1 isoform X1	Alt a 7	P42058	<i>A. alternata</i> (alternaria)	71/55	1.68×10^{-56}	NADPH-dependent FMN reductase	AF053	inhalation	7
XP_022853324.1	60S acidic ribosomal protein P2B-like	Pru du 5	Q8H2B9	<i>P. dulcis</i> (almond)	95/85	1.94×10^{-31}	60S acidic ribosomal protein	AF070	ingestion	1

Table 1. continued

accession no. ^b	protein name	homologous allergen in the IUIS database	UniProt code	organism	amino acid similarity/identity (%)	E value	allergen family name	AllFam code	route of sensitization	total potential olive pollen allergens identified in the family ^c
XP_022854082.1	luminal-binding protein S-like	Cor a 10	Q9FSY7	<i>C. avellana</i> (common hazel)	97/92	0	heat shock protein HSP70	AF002	ingestion	22
XP_022897418.1	peroxiredoxin-2	Mala f 3	P56578	<i>M. furfur</i>	60/45	5.90×10^{-44}	redoxin	AF131	inhalation	5
XP_022855565.1	soluble inorganic pyrophosphatase 6, chloroplastic	Der f 32	A0A088SCP3	<i>D. fariniae</i> (human dust mite)	67/47	2.04×10^{-74}	inorganic pyrophosphatase	AF205	inhalation	1
XP_022885711.1	thioredoxin H-type	Tri a 25	Q9LDX4	<i>T. aestivum</i> (wheat)	76/57	9.12×10^{-47}	thioredoxin	AF023	ingestion	5
XP_022875019.1	actin-depolymerizing factor -like	Der f 31	A0A088SAV1	<i>D. fariniae</i> (human dust mite)	59/38	1.32×10^{-23}	actin-depolymerizing factor-homologue	AF201	inhalation	6
XP_022889510.1	ferritin-2, chloroplastic-like isoform X2	Der f 30	L7UZ91	<i>D. fariniae</i> (human dust mite)	56/39	1.70×10^{-19}	ferritin	AF198	inhalation	1
XP_022851627.1	aldehyde dehydrogenase family 2 member B7, mitochondrial-like	Cla h 10	P40108	<i>C. herbarum</i>	68/56	2.04×10^{-74}	aldehyde dehydrogenase	AF040	inhalation	5
XP_022867276.1	aspartic proteinase-like	Aed a 11	Q03168	<i>A. aegypti</i> (yellow fever mosquito)	72/44	1.28×10^{-84}	eukaryotic aspartyl protease	AF004	contact	2
XP_022896740.1	peptidyl-prolyl-cis-trans isomerase 1	Hev b Cyp	F8RW92	<i>H. brasiliensis</i> (latex)	97/91	1.58×10^{-109}	cyclophilin	AF038	inhalation/contact	7
XP_022855094.1	ribosomal protein L3-2	Asp f 23	BOXSL2	<i>A. fumigatus</i>	73/69	1.90×10^{-105}	ribosomal protein L3	AF058	inhalation	1
XP_022895071.1	glyceraldehyde-3-phosphate dehydrogenase, cytosolic	Tri a 34	C7C4X1	<i>T. aestivum</i> (wheat)	95/87	5.90×10^{-101}	glyceraldehyde-3-phosphate dehydrogenase	AF184	inhalation	7
XP_022882931.1	subtilisin-like protease SPT4.15	Cuc m 1	Q39547	<i>C. melo</i> (melon)	64/47	0	subtilisin-like serine protease	AF021	ingestion	5
XP_022862800.1	malate dehydrogenase, mitochondrial	MDH cit 1	P17783	<i>C. lanatus</i> (watermelon)	97/86	0	lactate/malate dehydrogenase	AF014	ingestion/inhalation	9
XP_022874613.1	malate dehydrogenase, cytosolic	-	(37) ^c	<i>F. excelsior</i> (ash)	-	-	-	-	-	-
XP_022877964.1	probable serine/threonine-protein kinase PBL23	Sal k 2	Q8L5K9	<i>S. kali</i> (Russian thistle)	60/36	3.73×10^{-53}	protein kinase	AF017	inhalation	2
XP_022876859.1	reactive intermediate deaminase A, chloroplastic-like	Der f 34	A0A1J1DL12	<i>D. fariniae</i> (human dust mite)	58/30	8.23×10^{-18}	RidA/YigF/L-PSP family	AF213	inhalation	1
XP_022847291.1	stress-related protein-like	Hev b 3	F8SKC3	<i>H. brasiliensis</i> (latex)	66/50	3.67×10^{-58}	rubber elongation factor	AF125	contact	2
XP_022891594.1	aldehyde oxidase GLOX-like	Art an 7	A0A2H4HHY6	<i>A. annua</i> (sweet wormwood)	57/39	2.27×10^{-132}	galactose oxidase	-	inhalation	1
XP_022843917.1	cytochrome c	Cur l 3	Q96VP3	<i>C. lunata</i>	78/56	1.77×10^{-44}	cytochrome c	AF006	inhalation	2
XP_022889919.1	elongation factor 1-delta	Pen c 24	Q69BZ7	<i>P. citrinum</i>	78/40	3.83×10^{-24}	eukaryotic elongation factor 1	AF011	inhalation	2
XP_022844073.1	serpin-ZX-like	Tri a 33	P93692	<i>T. aestivum</i> (wheat)	72/55	1.34×10^{-144}	serpin serine protease inhibitor	AF018	inhalation	1
XP_022891403.1	3-oxoacyl-[acyl-carrier-protein] reductase 4-like	Alt a 8	P0C0Y4	<i>A. alternata</i> (alternaria)	55/32	1.82×10^{-30}	short chain dehydrogenase	AF028	inhalation	3

Table 1. continued

accession no. ^b	protein name	homologous allergen in the IUIS database	UniProt code	organism	amino acid similarity/identity (%)	E value	allergen family name	Allfam code	route of sensitization	total potential olive pollen allergens identified in the family ^c
XP_022890941.1	triosephosphate isomerase, cytosolic-like, partial	Tri a 31	Q9FS79	<i>T. aestivum</i> (wheat)	91/80	0	triosephosphate isomerase	AF032	inhalation	4
XP_022863167.1	vicilin-like seed storage protein At2g28490, partial	Coc n 1	A0A053B0K0	<i>C. nucifera</i> (coconut tree)	70/52	2.03×10^{-108}	cupin	AF045	ingestion	1
XP_022856299.1	major allergen Pru ar 1-like	Pru ar 1	OS0001	<i>P. armeniaca</i> (apricot tree)	79/67	1.46×10^{-63}	Bet v 1 family	AF069	ingestion	1
XP_022861507.1	eukaryotic translation initiation factor 3 subunit 1-like	For t 2	B2ZPG7	<i>F. taiwanica</i> (biting midge)	68/48	7.37×10^{-112}	WD-40 repeat	AF142	contact	1
XP_022899451.1	fructose-bisphosphate aldolase, cytoplasmic isozyme-like	Sal s 3	C0H911	<i>S. salar</i> (Atlantic salmon)	74/60	2.48×10^{-145}	fructose bisphosphate aldolase class I	AF197	ingestion	5
XP_022874390.1	acid β -fructofuranosidase 2, vacuolar-like	Sola 1.2	Q8RVW4	<i>S. lycopersicum</i> (tomato)	80/60	0	glycoside hydrolase family 32	AF052	ingestion	3
XP_022889771.1	calnexin homologue 1-like	Pen ch 31	Q2TLL9	<i>P. chrysogenum</i>	67/44	5.76×10^{-93}	calreticulin	AF055	inhalation	6

^aMS/MS spectra for the five proteins with single peptide entries in the three biological replicates (glutathione S-transferase L3-like, isoflavone reductase-like protein, peptidyl-prolyl *cis-trans* isomerase CYP21-like, pollen allergen Che a 1-like, and superoxide dismutase [Cu–Zn] 2-like isoform X3) are shown in Figure S4. ^bNCBI accession numbers of the *Olea europaea* var. *syvestris* proteins sharing the highest sequence identity with Allergome allergens of the indicated allergen families (see also Table S7). ^cOle e 7 sequence has been recently published and is not yet deposited in UniProt. ^dCytosolic malate dehydrogenase from ash (*F. excelsior*) pollen, a member of the Oleaceae family, was suggested as a potential allergen in ref 39.

PAGE, appears as two main bands of 17 to 18.5 kDa for the nonglycosylated and N-glycosylated isoforms, respectively, with a pI interval (5.0 to 9) for the different isoforms of this allergen. On the contrary, a theoretical mass of 18.1 kDa and 8.7 pI were predicted for natural Ole e 15, which led us to hypothesize that the presence of Ole e 1 could have masked the identification of Ole e 15 in the extract.

To address this question, WB analysis after 1D and 2D SDS-PAGE of the olive pollen extract with anti-Ole e 1 and anti-Ole e 15 pAb and IgE inhibition assays confirmed that nOle e 15 is present as a monomeric protein with an experimental pI of ~8.4 and an apparent molecular mass very similar to that of glycosylated Ole e 1 (Figure SA,B). As a consequence, the reactivity to nOle e 15 in a cosensitized patient with reactivity to Ole e 1 would remain hidden because of the high protein content of Ole e 1 and its prevalence and might be detected only if the serum was previously inhibited with Ole e 1 (Figure 5C).

DISCUSSION

Olive pollen is an important allergenic source in those regions with extensive cultivars for olive oil production. The increasing cultivation of this tree in China, India, Australia, Russia, California (United States), and multiple countries of South America has led to a growing number of olive-pollen-allergic patients. Therefore, it will become a top allergenic health problem worldwide in the next years, and thus the definition of its allergenome will significantly improve the clinical management of olive-pollen-allergic patients.

Here, to delineate the cultivated olive pollen proteome and its allergenome, we took advantage of the recently reported wild olive genome in combination with a high-performance bottom-up LC–MS/MS approach to optimize protein identification. The number of sequences obtained represents the 3.8% of the complete genome-predicted proteome and is in agreement with published data regarding transcriptomic analysis in olive pollen, in which 1976 transcripts encoding complete proteins, including isoforms, were found.⁴⁴ Then, we performed a functional annotation of the identified proteins, using a newer version of Blast2GO software and a more up-to-date Gene Ontology (GOA) database than previously used for the genome.¹⁴ Our annotation results are as expected for pollen protein functions in germination and tube growth, extremely regulated multistep processes in which secreted or membrane proteins and enzymes play a major role.⁴⁵ In fact, they are also in concordance with other pollen proteomics studies in which most of the proteins were annotated to metabolic processes.^{46–49} Collectively, we have performed a comprehensive in-depth proteomics study of the olive tree allergenic pollen and elucidated the distribution of its proteins according to their associated biological function and location, which can be helpful for further studies related to olive pollen biology. Nevertheless, it must be considered that the obtained data are highly dependent on technical and biological issues. For instance, it is known that protein extraction methods in plant proteomics can be determinant for detecting hydrophobic membrane proteins and low abundant proteins.^{50,51} In addition, it has been reported that the use of different solid-phase extraction methods (i.e., ZipTip vs SOLAμ) after in-gel trypsin digestion and prior to nLC–MS/MS analysis can increase the coverage in the proteome.⁵² Moreover, previous studies have demonstrated that pollen grains from different olive cultivars differ in the quantity and number of proteo-

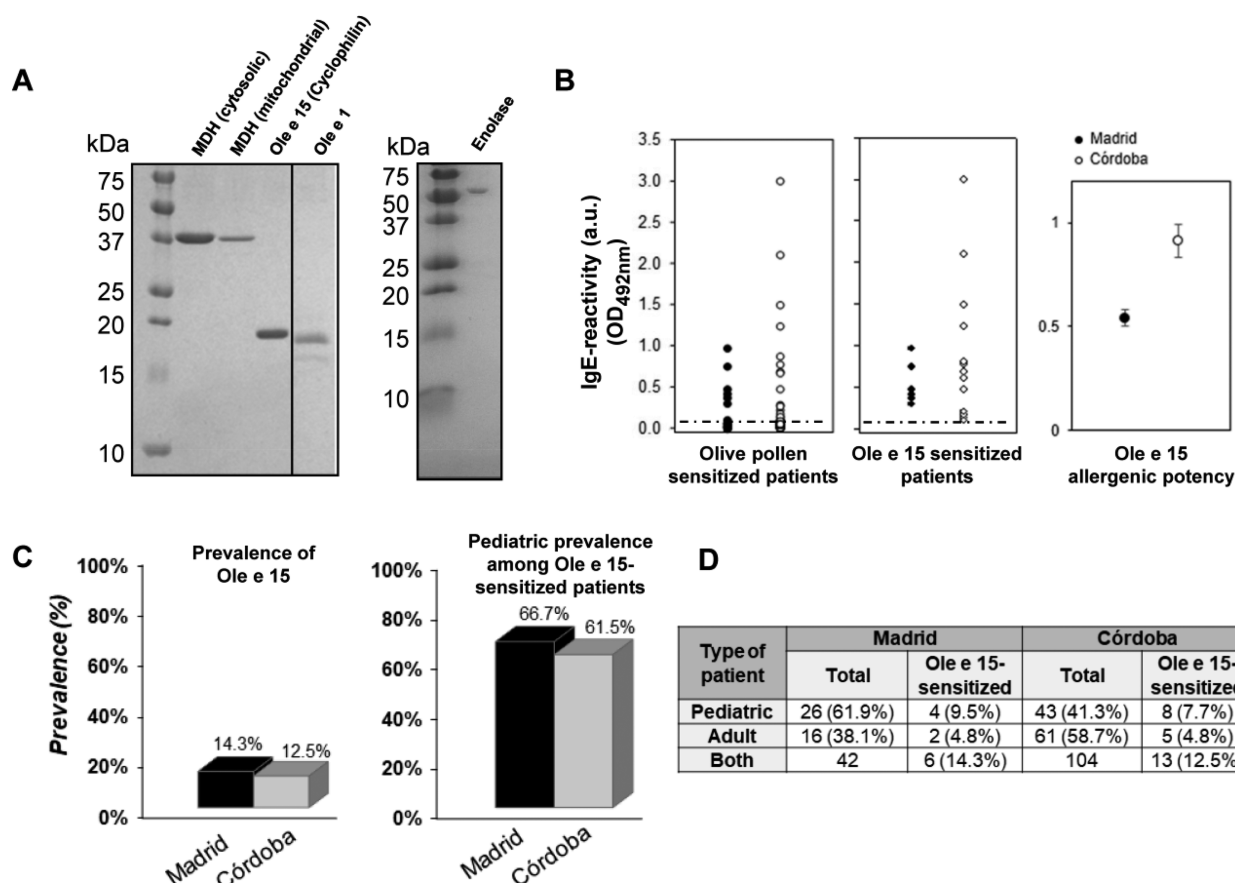


Figure 3. Analysis of the purified recombinant proteins and IgE reactivity to rOle e 15. (A) Analysis of 0.5 μ g of purified recombinant proteins by Coomassie Blue staining after 15% SDS-PAGE under reducing conditions. Purified natural Ole e 1 was used as the control. (B) Dot plots representing the strength of the IgE reactivity to rOle e 15. The dotted line indicates the response threshold (O.D. values >0.1). (C) Bar graphs representing the prevalence of Ole e 15-sensitized patients in both populations (left) and the pediatric prevalence among rOle e 15-sensitized patients (right). (D) Prevalence data are also displayed.

forms, including those of important allergens like Ole e 1, Ole e 2, and Ole e 5.^{53–55} This, together with environmental factors like geographic variability in the reached pollen counts, the coexistence of other allergen sources and pollution, as well as human factors like diet, alter the sensitization patterns to olive pollen, especially regarding minor allergens.⁵⁶ Therefore, further proteomics studies analyzing pollen grains from common cultivars using alternative protein extraction methods would add relevant biological and clinical information to the data presented here.

We focused our study on the identification of allergenic proteins in olive pollen to complete its allergenome. From the 76 allergen families present in the genome-predicted proteome, 47 allergen families were identified in pollen, with 20 not previously described in this source. Importantly, all of the previously reported allergen families were found.^{17–19,57} Only the allergen Ole e 6 was not observed. Ole e 6 is present among the potential allergens identified from the genome-predicted proteome (Table S6), and its absence in our proteomics data could be due to either its extremely low abundance in pollen⁵⁸ or the stringent filters used.

An important feature for some allergenic sources is the high degree of heterogeneity in the same allergen family. According to the WHO/IUIS guidelines for allergen nomenclature, allergenic proteins from the same species are considered isoallergens when they have similar molecular size and a sequence identity of at least 67%, and they are considered

allergen variants (isoforms) when the proteins display a sequence identity >90%. It has been shown that isoallergens and allergen variants found in pollen can display different IgE reactivities.^{19,38,53–55,59} Moreover, in some sources, proteins of the same allergen family, but that are not considered isoallergens, can be allergenic.⁶⁰ In our study, we found more than one protein in 34 of the 47 identified allergen families. In this sense, the Ole e 1-like allergen family was the most heterogeneous (Table 1 and Table S7). Of the 17 proteins found in this family, eight were Ole e 1 variants, and five were Ole e 1 isoallergens. This high degree of Ole e 1 polymorphism had already been described and can vary among different olive cultivars.^{55,61} The further study of these allergen polymorphisms might help us to identify hypoallergenic molecules, which can be used for the development of safer immunotherapy strategies. On the contrary, we observed interesting potential allergens that remain to be studied. For example, the presence of a thaumatin-like protein (TLP) similar to Act d 2 was found (Table 1 and Table S7). A member of the TLP family had been described as an olive fruit allergen (Ole e 13),⁶² but the presence of other TLPs in olive pollen was unknown. Thus, this finding suggests that this protein family could also be allergenic in olive-pollen-allergic patients and may be implicated in cross-reactivity with fruits. Additionally, we observed the presence of several allergen families commonly found in the pollen of weeds, grasses, and trees,⁶³ such as pectate lyase, papain-like cysteine protease, and

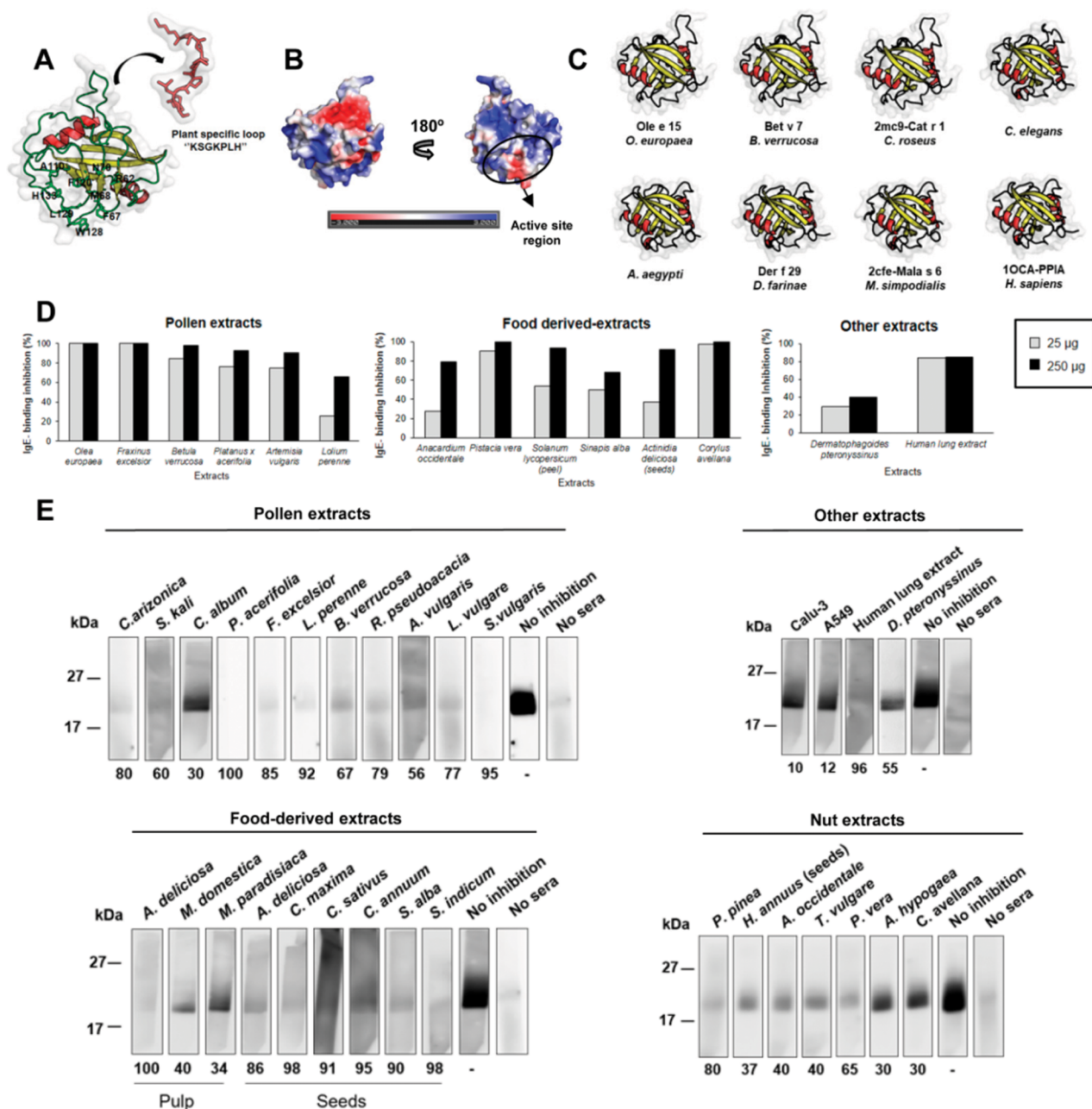


Figure 4. Prediction and comparison of the Ole e 15 structure with other cyclophilins and analysis of its potential IgE cross-reactivity. (A) 3D model of Ole e 15 structure using that of Cat r 1 (PDB: 2MC9) as a template. Putative catalytic residues (R62, F67, M68, N70, A110, F120, W128, L129, and H133) and the divergent loop (KSGKPLH) sequence are labeled. (B) Predicted electrostatic potential of Ole e 15 using the Adaptive Poisson–Boltzmann Solver (APBS) program, considering 2 and 78 as dielectric constant values for protein and solvent, respectively. The active-site region is highlighted with a circle. Electropositively and electronegatively charged regions are colored blue and red, respectively. Neutral regions are colored white. (C) Comparison of the structures of plant (Ole e 15, Bet v 7, and Cat r 1), fungal (Mala s 6), and animal (human PPIA, Der f 29, *C. elegans*, and *A. aegypti*) cyclophilins. Plant and animal cyclophilins were predicted using structures of Cat r 1 (PDB: 2CM9) and human PPIA (PDB: 1OCA) as Expasy Swiss-Model tool templates, respectively. All structures were generated by PyMOL. (D) Analysis of the IgE inhibition to rOle e 15 was performed by ELISA using an equivolumetric sera pool of olive pollen patients and 25 and 250 μ g of the indicated extracts as inhibitors. (E) Analysis of the IgE binding to Ole e 15 was performed by WB using an equivolumetric sera pool and 500 μ g of the indicated protein extracts as inhibitors.

cyclophilin. These data validated our workflow to identify potential allergenic proteins, defining the most complete allergenic proteomics profile of olive pollen up to date.

The next goal of this work consisted of the validation of the allergenic potential of some of the newly identified candidate allergens. We selected those protein families reported to be allergenic in at least two allergenic sources, including one

nonpollenic, to identify those olive pollen allergens implicated in cross-reactivity. MDH (cytoplasmic and mitochondrial), enolase, and cyclophilin were selected for validation. After their cloning, expression, and purification, only cyclophilin was able to bind IgEs from the allergic patients tested. Cyclophilin was named Ole e 15 according to the WHO/IUIS. Remarkably, cytoplasmic and mitochondrial MDHs had been previously

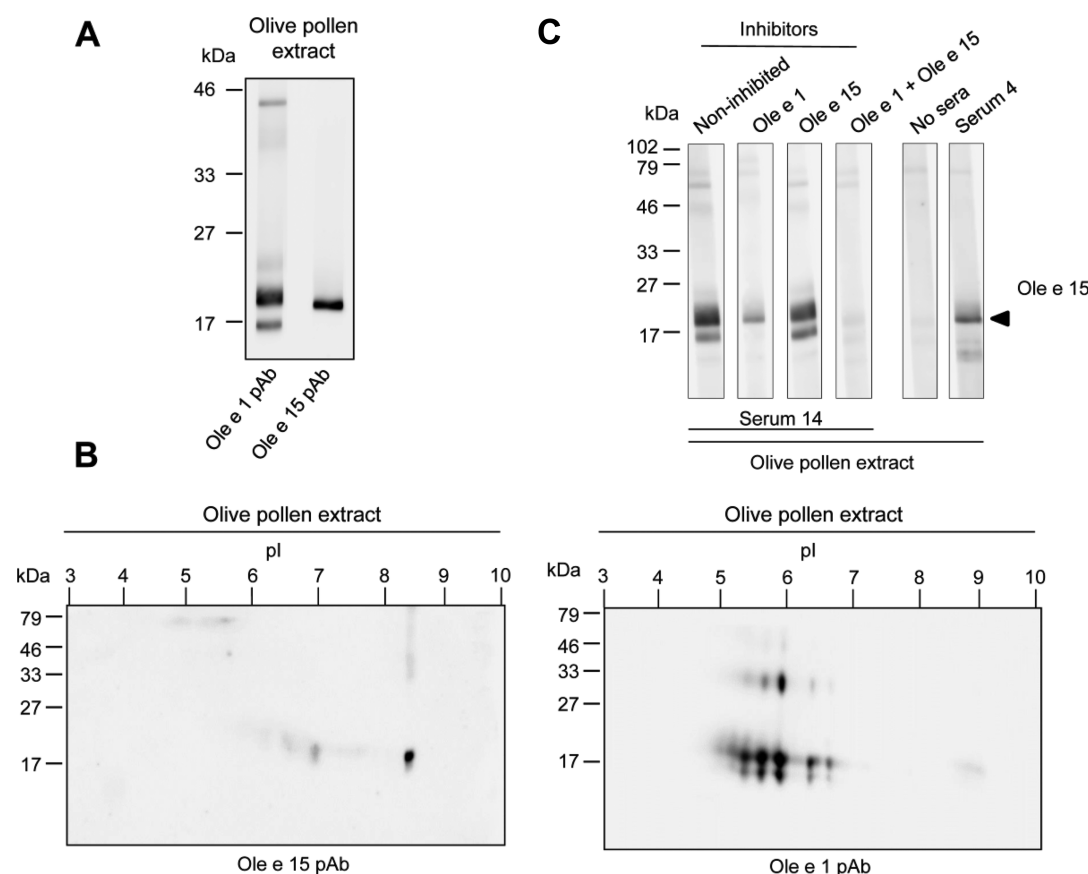


Figure 5. Analysis of Ole e 15 in the olive pollen extract. WB analysis after SDS-PAGE of olive pollen extract (40 μ g) using anti-Ole e 1 and anti-Ole e 15 pAbs: (A) 1D SDS-PAGE and (B) 2D SDS-PAGE. (C) IgE reactivity analysis after SDS-PAGE and transference to membranes of olive pollen extract. Strips were incubated with serum no. 14, sensitized against Ole e 1 and Ole e 15, after either no inhibition or its IgE inhibition with alternatively 10 μ g of purified nOle e 1, rOle e 15, or both. No signal was detected without serum incubation (No sera). The IgE reactivity of a serum sensitized to Ole e 15 but not to Ole e 1 was also analyzed (serum no. 4).

described as potential allergenic proteins in watermelon, soybean seeds, ash pollen, and yeast; enolase in latex, ragweed pollen, and molds; and cyclophilin in carrot, tomato, birch pollen, molds, and dust mites.^{9,39,43,64–72} Therefore, it cannot be discarded that these olive pollen proteins could be reported as allergenic if tested against other allergic populations or if they were expressed in heterologous systems different from *E. coli*, which would provide post-translational modifications and complex folding.

The last objective of our study was the characterization of olive pollen cyclophilin. The recombinant protein structure exhibited a folding with similar secondary structure as Cat r 1 by far-UV CD,⁴⁰ indicating that the protein was correctly folded. Regarding its immunological behavior, we observed that the prevalence of Ole-e-15-sensitized patients was ~13% among olive-pollen-allergic patients from Córdoba and Madrid, with the majority of them showing high IgE levels against the allergen. Although Ole e 15 sensitization was more frequent in pediatric patients (17.4%) than in adults (7.8%) in both populations, the correlation between Ole e 15 sensitization and pediatric patients was not statistically significant. However, this trend will be assessed in further studies with higher cohorts. After inhibition experiments, we found that Ole e 15 is highly implicated in cross-reactivity with pollen, plant-derived food, and dust mites. Human cyclophilin, which shows 70% sequence identity with Ole e 15, was also able to inhibit the IgE binding to the recombinant protein. Our

results are in agreement with a previous study in which plant cyclophilins were predicted to cross-react with animal cyclophilins due to a high degree of surface conservation.⁴⁰ This confirmed the broad cross-reactivity of cyclophilin and extended the physicochemical and immunological knowledge of this pan-allergen to olive pollen. Moreover, because Ole e 15 shares similar physicochemical properties with Ole e 1, the identification of cyclophilin as an allergen has remained elusive by standard techniques. Therefore, the in-depth olive pollen proteomics analysis allowed the identification of this relevant allergen and suggests that its presence should be assessed in natural preparations to prevent the overestimation of the number of patients allergic to Ole e 1.

In summary, we have performed the most comprehensive proteomics profiling of olive pollen and defined its allergen families. We have also unmasked Ole e 15, a relevant olive pollen allergen belonging to the cyclophilin family with similar physicochemical features as the main olive pollen allergen. After cloning, expression, and characterization of Ole e 15, we found that it is a highly potent cross-reactive allergen with plant and animal cyclophilins.

■ ASSOCIATED CONTENT

§ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jproteome.9b00167.

Table S1. Information on the olive-pollen-allergic patients used in the study. Table S2. List of the oligonucleotides used in the study. Table S8. Information on the potential allergens cloned. Figure S1. Summary of the LC–MS/MS olive pollen identified proteins in the three replicates. Figure S2. Number of GO terms annotated of biological process, molecular function, and cellular component. Figure S3. KEGG pathways and GO terms enrichment. Figure S4. Mass spectra corresponding to proteins identified with a single peptide. Figure S5. CD analyses of rOle e 15. Figure S6. Amino acid sequence alignment of Ole e 15 with other allergenic cyclophilins ([PDF](#))

Table S3. Proteins identified in olive pollen by LC–MS/MS ([XLSX](#))

Table S4. Olive pollen proteins annotated with GO terms ([XLSX](#))

Table S5. Olive pollen proteins predicted as secreted ([XLSX](#))

Table S6. Wild olive genome predicted proteins belonging to allergen families ([XLSX](#))

Table S7. Olive pollen proteins identified by LC–MS/MS belonging to allergen families ([XLSX](#))

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Notes

The authors declare no competing financial interest.

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository, with the data set identifiers PXD012334 (Replicate 1), PXD012279 (Replicate 2), and PXD012280 (Replicate 3).

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