



Pattern of sensitization to *Juniperus oxycedrus* 4EF-hand polcalcin, Jun o 4, compared with the 2EF-hand grass homolog Phl p 7 in a general Italian population of subjects suffering from pollinosis

Teresa Ricciardi^{a,b,1}, Ivana Giangrieco^{a,b,*,1}, Claudia Alessandri^{b,c}, Chiara Rafaiani^c, Lisa Tuppo^{a,b}, Maurizio Tamburrini^a, Anna Filomena Digilio^d, Maria Antonietta Ciardiello^a, Adriano Mari^{b,c}

^a Institute of Biosciences and BioResources (IBBR), CNR, Naples, Italy

^b Allergy Data Laboratories (ADL), Latina, Italy

^c Associated Centers for Molecular Allergology (CAAM), Rome, Italy

^d Research Institute on Terrestrial Ecosystems (IRET), CNR, Naples, Italy.

ARTICLE INFO

Keywords:

Cupressaceae
IgE binding
Jun o 4
Polcalcin
Pollen allergy
Cypress allergy

ABSTRACT

Cupressaceae pollen causes allergic reactions worldwide with long-lasting symptomatic periods. Currently, no cypress polcalcin is available for diagnostic purposes. With the aim to investigate the pattern of sensitization to a cypress polcalcin, a synthetic gene of Jun o 4, the *Juniperus oxycedrus* 4EF-hand polcalcin, was cloned and expressed in *Escherichia coli*. Its features were investigated in comparison with the grass 2EF-hand Phl p 7. Rhinitis was the symptom most frequently reported in a cohort of Italian patients sensitized to rJun o 4 and/or rPhl p 7. The detection of many pollen allergic patients sensitized to the cypress polcalcin, but negative to Phl p 7, indicates that Phl p 7 cannot be further considered a marker of sensitization towards all the polcalcins. A 4EF-hand cypress polcalcin claims the inclusion in allergy diagnostic tests. In addition, the sensitivity of polcalcins to gastrointestinal digestion is reported and discussed for the first time.

1. Introduction

Pollen allergies constitute a common cause of type I hypersensitivity worldwide. In general, an increase of allergic symptoms due to tree, weed and grass pollens has been observed during the last years. In industrialized countries, allergic reactions towards pollens can affect up to 30% of populations [1]. Pollen allergens belong to different protein families [2]. They include pathogenesis related (PR) proteins involved in plant defense mechanisms, such as PR-2 (endo 1,3-glucanases), PR-5 (thaumatin-like proteins, TLP), PR-10 (Bet v 1-like proteins), PR-12 (defensins) and PR-14 (lipid transfer proteins, LTP). Additional pollen allergens have structural roles (expansins and profilins), or enzyme function (ribo-nucleases, pectate lyases and polygalacturonases) or other functions (calcium-binding proteins, CBP).

CBP are ubiquitous proteins found in all eukaryotic organisms. These proteins can be classified in different subfamilies and some of them include allergenic components [3]. CBP of different subfamilies show

different molecular masses and low overall amino acid sequence identity. Nevertheless, they share a conserved domain, named the EF-hand motif, consisting of a 12 residue calcium-binding loop flanked on both sides by α -helices (helix-loop-helix motif) [4,5]. CBP play an important biological role associated with the presence of this structural motif and its ability to bind and transport calcium ions.

Polcalcins (pollen calcium-binding proteins) constitute a CBP subfamily specifically found in tree, grass and weed pollens [6,7]. They are considered important respiratory allergens, although are reported as minor allergens [8]. In plants, polcalcins are preferentially expressed in anthers and in mature pollen [9] and might be involved in cellular processes influencing the pollen reproductive function [10]. According to the number (2, 3 or 4) of EF-hand motifs contained in their primary structure, the allergenic polcalcins can be classified into three groups. Allergenic polcalcins with 2EF-hand motifs have been detected in the pollen from many species, such as timothy grass, birch, alder, ambrosia, mugwort, goosefoot, bermuda grass, ash, olive tree and lilac. Allergenic

* Corresponding author at: Institute of Biosciences and BioResources (IBBR), CNR, Naples, Italy.

E-mail address: ivana.giangrieco@ibbr.cnr.it (I. Giangrieco).

¹ These authors equally contributed to the study.

polcalcins with 3EF-hands motifs were found in the pollen from ambrosia and birch, whereas examples containing 4EF-hands have been detected in prickly juniper, cypress and olive [11].

Literature reports suggest that the major IgE epitopes of CBP allergens are associated with the less conserved portions of the proteins, rather than with the highly conserved calcium-binding regions [12]. Despite the sharing of the EF-hand motif, there is no evidence for IgE cross-reactivity between CBP allergens belonging to different sub-families, and between plants, animals and humans. Three polcalcins belonging to the 2EF-hand group, namely Phl p 7 and/or Bet v 4 and/or Aln g 4, are currently used by the most widespread allergy diagnostic systems. Phl p 7 belongs to the Poaceae, whereas Bet v 4 and Aln g 4 to the Betulaceae plant family. 3EF-hand and 4EF-hand polcalcins are not available and currently not used for allergy diagnosis. Therefore, some sensitizations to the pollen polcalcins of many plants could escape the detection due to the lack for diagnosis of the specific molecules. For instance, Cupressaceae sensitization is one of the most important causes of pollinosis worldwide, but no polcalcins of this plant family are now available for diagnostic purposes.

Cypress is a common name given to various coniferous trees belonging to the Cupressaceae family. Among them, those most important for allergic diseases are included in five different genera, namely *Cupressus*, *Hesperocyparis*, *Juniperus*, *Cryptomeria* and *Chamaecyparis* [13]. Except for Antarctica, species of this family are found in all of the Earth's continents. Moreover, as different Cupressaceae species pollinate in different periods, the allergy symptomatic period is long-lasting. A progressively increase in the prevalence of cypress allergy has been reported [13] and it can be connected to different factors, including pollution and the effect of global warming inducing a re-distribution of allergenic species towards northern areas. Therefore, since allergy to cypress pollen is widely distributed, and it is reported as highly prevalent and capable to cause severe pollinosis, its careful diagnosis has a high relevance for the management of sensitized patients. In Italy, most of Cupressaceae pollen is present in the atmosphere between January and mid-April, but a second huge pollination can occur in November, caused by *Juniperus oxycedrus* (Juniperioideae subfamily) growing along the coast of almost all the Mediterranean countries and in the internal woody areas [14].

Allergenic polcalcins from Cupressaceae reported so far include Jun o 4 (formerly Jun o 2) and Cup a 4, both belonging to the group containing 4EF-hand motifs. Jun o 4 was identified in 1998 [15] and described as a *J. oxycedrus* pollen allergen cross-reacting with taxonomically related and unrelated species. Later, Cup a 4 was identified as an allergenic polcalcin of *Cupressus arizonica* pollen [16]. High sequence identity between Jun o 4 and Cup a 4 was observed. Similar to some other polcalcins, such as Phl p 7, the characterization of Jun o 4 [15] and Cup a 4 [16] was obtained by producing the recombinant molecules since these proteins are contained in very low amount in the natural source and their isolation seems not possible.

Recombinant Jun o 4 (rJun o 4) had been produced in *Escherichia coli* and characterized some years ago [15]. This study demonstrated that *J. oxycedrus*, *Juniperus ashei*, *C. arizonica*, *Cupressus sempervirens*, *Parietaria judaica*, *Olea europaea*, and *Lolium perenne* pollen extracts were able to inhibit the IgE binding to blotted rJun o 4 at different concentrations. The structural characterization showed that Jun o 4 has a molecular mass approximately doubled with respect to Phl p 7; moreover, it shows a double globular-domain, each one consisting of a 2EF-hands motif [17]. In addition, this study revealed the presence of IgE cross-reaction between the 4EF-hands Jun o 4 and the 2EF-hands Phl p 7, indicating sharing of IgE epitopes. The group of patients with multiple pollen allergies analyzed in this study showed the following hierarchy of IgE cross-reactivity among the families of calcium-binding allergens: rPhl p 7 > rAln g 4 > rJun o 4 > rBet v 3. Therefore, these results suggested that rPhl p 7 was the EF-hand allergen containing most IgE epitopes and that it could represent a diagnostic marker. However, the pattern of sensitization to Jun o 4 and Phl p 7 in the groups of patients analyzed in those

early studies was unclear, mainly due to the limited number of serum samples and the lack of comparison with other allergens. In the present study, we sought to address this gap and investigate the pattern of sensitization of the cypress Jun o 4 in comparison with the grass Phl p 7. Therefore, the prickly juniper Jun o 4 was produced as a recombinant molecule in *E. coli* and characterized. The prevalence of sensitization to this polcalcin was then investigated by testing its IgE-binding capacity in a large general Italian population. In addition, to investigate the possibility that ingested polcalcin-containing pollens or honey may cause severe allergic reactions, like stable food allergens, or that they may cause mild symptoms, like other allergens which are degraded by the gastrointestinal proteases, digestibility experiments were performed.

2. Materials and methods

2.1. Materials

Jun o 4 gene was synthesized by GeneArt AG (Regensburg, Germany). This gene was designed in order to have a protein with 100% identity with the sequence of Jun o 4 reported in UniProtKB with the accession number O64943. rPhl p 7, expressed in *Escherichia coli*, was purchased from Indoor Biotechnologies Ltd. (Cardiff, UK). The proteases, namely porcine pepsin, porcine trypsin and bovine chymotrypsin were from Roche Diagnostics GmbH (Mannheim, Germany).

2.2. Cloning, expression and purification of rJun o 4

A synthetic Jun o 4 gene was inserted into a pRSET A expression vector between the restriction sites BamHI and XhoI. This plasmid (pRSET A – rJun o 4) encodes for the Jun o 4 protein with an N-terminal polyhistidine tag. *E. coli* BL21(DE3) cells were transformed with the pRSET A – rJun o 4 plasmid and grown in LB medium, containing 50 µg/ml ampicillin, at 37 °C, to an optical density of approximately 0.6 at 600 nm. Then the expression was induced with IPTG to a final concentration of 1 mM. After induction, the cells were incubated at 37 °C overnight and then harvested and resuspended in Lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole pH 8), 1 mg/ml lysozyme and 1 mM PMSF. After 30 min on ice, the cells were disrupted by sonication and the soluble protein fraction was separated from the cell debris by centrifugation at 10,000 ×g for 30 min, at 4 °C. Next, the recombinant protein was purified by affinity chromatography using a Ni²⁺-charged resin according to the manufacturer's instructions (Qiagen, Hilden, Germany): the cleared lysate was mixed with Ni-NTA agarose (nickel-nitrilotriacetic acid resin) to allow the binding between the 6xHis-tagged proteins and the matrix, and then loaded onto a column, according to the Qiagen protocol. After appropriate washing of the column, the bound proteins were eluted with elution buffer (50 mM NaH₂PO₄, 300 mM NaCl and 250 mM imidazole pH 8), prepared according to the manufacturer's instructions. The fractions containing rJun o 4 were detected by SDS-PAGE analysis, pooled and finally dialyzed against 10 mM phosphate buffer pH 7.4. The purity of the recombinant protein was assessed by SDS-PAGE analysis and N-terminal amino acid sequencing.

2.3. Estimation of the protein concentration

The protein concentration in the lysate was estimated with the BIO-RAD Protein Assay (Bio-Rad, Milan, Italy), using calibration curves made with bovine serum albumin.

The estimation of the purified protein concentration was obtained using the molar extinction coefficient at 280 nm (8730 M⁻¹ cm⁻¹) calculated on the basis of the amino acid sequence with the ProtParam algorithm on the ExPASy Bioinformatics Resource Portal (www.expasy.org).

2.4. Amino acid sequencing

The recombinant Jun o 4 protein (1 nmole) was subjected to automated direct sequencing of the N-terminal region with a Protein Sequencer PPSQ-33B (Shimadzu Corporation, Tokyo, Japan).

2.5. Separation on SDS-PAGE

The non-induced cell lysate of rJun o 4, induced cell lysate and purified protein were subjected to reducing 15% SDS-PAGE on a BIO-RAD mini-PROTEAN apparatus (Bio-Rad, Milan, Italy). Following electrophoresis, the proteins were stained with Coomassie Brilliant Blue (Bio-Rad, Milan, Italy).

2.6. Protein stability to the SGF and SIF conditions

In vitro simulated gastric digestion of rPhl p 7 and rJun o 4 was performed as previously described by Bernardi et al. [18]. Briefly, rJun o 4 and rPhl p 7 were solubilized in simulated gastric fluid (SGF), made with 0.15 M NaCl adjusted to pH 2 with 1 M HCl, before the addition of pepsin at an enzyme/substrate ratio of 1:20 w/w. The digestion was performed at 37 °C. Protein aliquots were taken at zero and 120 min and analyzed by RP-HPLC. The digestion was stopped by raising the pH to 7.4 with the addition of 50 mM sodium phosphate buffer pH 7.4.

In vitro simulated intestinal digestion was performed according to the United States Pharmacopeia, as described by Ciacchi et al. [19]. Briefly, proteins were solubilized in simulated intestinal fluid (SIF), consisting in 0.05 M potassium phosphate buffer pH 6.8 containing trypsin and chymotrypsin, both at an enzyme:substrate ratio of 1:50 w/w. The digestion was performed at 37 °C. Aliquots were taken at zero and 120 min, and analyzed by RP-HPLC.

2.7. Analysis by RP-HPLC

Two aliquots of each of the digested samples of rJun o 4 and rPhl p 7, taken at time 0 and 120 min, were analyzed by RP-HPLC on a Vydac (Deerfield, IL) C8 column (0.21 × 25 cm), using a Beckman System Gold apparatus (Fullerton, CA). Elution was made by a multistep linear gradient of eluent B (0.08% TFA in acetonitrile) in eluent A (0.1% TFA) at a flow rate of 1 ml/min. The eluate was monitored at 220 and 280 nm.

2.8. Specific IgE detection by the FABER® multiplex testing system

The FABER® test (ADL s.r.l., Latina, Italy) is a multiplex in vitro serological test that allows the detection of specific IgE antibodies from allergic subjects [20–22]. It is a registered in vitro diagnostic (IVD) test which guarantees the presence of the essential quality requirements for in vitro diagnostics, including the absence of interferences caused by haemolysis, high levels of triglycerides, limit of detection, precision and repeatability, diagnostic accuracy, high levels of total IgE or by allergen-specific antibody isotypes, such as IgG. The FABER version used to perform the present study (FABER 244-122-122) bears 244 allergenic preparations from many different allergenic sources, representing 122 purified molecular allergens and 122 allergenic extracts, all conjugated to nanobeads [23]. The FABER biochip is used for an ELISA-like testing procedure that allows the detection of specific IgE to each of the 244 allergenic preparations, in addition to some experimental allergens, in a single run. The final signal for each allergen is obtained with the use of an optical scanner and elaborated with a specific software. The signals detected for each spot are interpolated with values obtained with an internal IgE standard reference curve present in each biochip and also used as a signal reference between chips. Signals intensities are then used to calculate the arbitrary units, FIU (FABER International Units), correlated with the level of IgE bound to each allergen spot.

The direct IgE detection was obtained using a set of biochips that included Jun o 4 as experimental molecule, in addition to extracts and

molecules routinely present on the FABER biochip. Before the immobilization on the FABER biochip, rJun o 4 was coupled to nanobeads in parallel with the standard allergens and following the same procedure applied to all the other allergenic preparations. The coupling was carried out by the same professionals which conjugate all the 244 allergens available on the FABER biochip. The quality of the coupling, and the amount of allergen bound to nanobeads, is always verified for each allergen, including the experimental ones, such as rJun o 4. As usual the quality of each batch of FABER biochips, bearing the spotted allergens, was verified before use. Therefore, the quality of rJun o 4 spotted on the FABER biochip was exactly the same as that of Phl p 7 and of all other spotted allergens.

2.9. Patients

The IgE profile of 8784 Italian patients consecutively tested with the FABER system was analyzed. A reliable clinical history was available only for patients which asked a medical examination directly to the allergologists of the Associated Centers for Molecular Allergology (CAAM), Rome. The symptoms reported by patients, and other details including the use of medications, were recorded in a specific questionnaire, as already described by [24]. All investigations were carried out according to the World Medical Association Declaration of Helsinki about Ethical Principles for Medical Research Involving Human Subjects. All patients gave their informed consent to the use of their clinical data in an anonymous form. They gave also informed consent to the use of their anonymized leftover serum for research purposes. Since the study was carried out within the routine activity, a formal approval by an external ethical committee was not required.

3. Results

3.1. Cloning, expression and purification of recombinant Jun o 4 (rJun o 4)

The sequence of Jun o 4 was cloned and expressed in *E. coli* as a soluble polyhistidine-tagged protein (Supplementary Fig. 1). After elution, the final expression product (rJun o 4) included 201 residues, which means that 36 additional residues (MRGSHHHHHHGMASMTGGQMGRLDYDDDDKDRWGS) were present at the N-terminus compared to the natural protein. This 36-residue sequence contained amino acids particular to the expression Vector

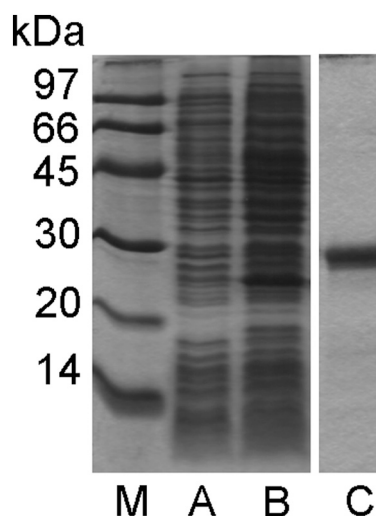


Fig. 1. SDS-PAGE analysis of the expression and purification of rJun o 4 produced in *E. coli* BL21 (DE3) cells. M, molecular mass markers; A, non-induced cell lysate; B, IPTG-induced cell lysate; C, affinity-purified rJun o 4.

(Xpress™Epitope, EK site) and the 6xHis tag. The purity of the rJun o 4 preparation was assessed with the SDS-PAGE (Fig. 1) and N-terminal amino acid sequencing. In line with literature reports [15], in SDS-PAGE the recombinant protein showed an apparent molecular mass of 29 kDa, although the value calculated on the basis of the amino acid sequence of the recombinant protein was 21.882 kDa (Fig. 1).

3.2. Comparative analysis of the amino acid sequence of Jun o 4 and that of other allergenic polcalcins

Fig. 2 shows the alignment of Phl p 7 (accession number O82040) with the first of the two globular domains, each one consisting of 2 EF-hand motifs, found in Jun o 4 (accession number O64943). The alignment obtained by the T-Coffee algorithm [25], on the ExPASy server, is in line with the results reported by Tinghino and collaborators [17] showing that Phl p 7 displays higher structural similarity with the first globular domain of Jun o 4, rather than with the second one.

The sequence of natural Jun o 4 comprises 165 residues (about 18 kDa) and contains 4EF-hand calcium binding motifs in positions 35–46, 71–82, 104–115 and 140–151 (UniProtKB). The sequence of natural Phl p 7 comprises 78 amino acids (about 9 kDa) and shows 2EF-hand motifs in positions 13–24 and 48–59. The sequence identity between Jun o 4 and Phl p 7 is 55% in the 76 residues of overlapping regions. The highest sequence identity has been registered for the EF-hand motifs, especially for the EF1-hand region (residues 35–46 in Jun o 4 and 13–24 in Phl p 7) where 10 residues of Jun o 4, out of 12, are conserved in Phl p 7 (83% identity) (Fig. 2). Six residues of the EF2-hand region of Jun o 4 (residues 71–82 in Jun o 4 and 48–59 in Phl p 7) are conserved in the overlapping region of Phl p 7.

The amino acid sequences of Jun o 4 and Phl p 7 were used as query sequences to perform similarity searches in the Allergome database using the Allergome Aligner tool and the Blast algorithm on the Allergome platform (www.allergome.org) [26]. The isoforms and isoallergens of Jun o 4 and Phl p 7 detected in the Allergome database are shown in Tables 1 and 2. These identity values were chosen because the WHO/IUIS Allergen Nomenclature Sub-Committee has ruled that sequences having between 67% and 90% identity to the original allergen are designated as isoallergens, whereas sequences having more than 90% identity are isoforms or variants [27]. Clearly, the level of expected co-recognition and cross-reaction between a protein under investigation and its isoforms is higher compared with its isoallergens. WHO/IUIS suggests that at 67% identity IgE cross-reactivity is possible, whereas at lower values the probability decreases [27].

Table 1 shows that the search carried out using Jun o 4 as query sequence detected in the Allergome database only one isoform, Cup a 4, found in *C. arizonica*. Like prickly juniper, this cypress belongs to the Cupressaceae family. Cry j polcalcin is an additional cypress polcalcin. It is found in *Cryptomeria japonica* and is reported as potential allergen (Allergome database) [26]. Its complete protein sequence, showing 76% of amino acids identical to Jun o 4, was retrieved from the EST database (Table 1). Table 2 shows the polcalcins detected by performing a similarity search using Phl p 7 as query sequence. On the basis of the rules fixed by WHO/IUIS Allergen Nomenclature Sub-Committee for classification of allergenic homologous proteins [27], six 2EF-hand polcalcins

were classified as Phl p 7 isoforms (sequences having more than 90% identity) and ten as isoallergens (between 67% and 90% identity). All the isoforms were from plant belonging to the Poaceae family. All the Phl p 7 isoallergens were from different plant families, namely Chenopodiaceae, Urticaceae, Oleaceae, Brassicaceae, Solanaceae and Betulaceae. Sequence identity values lower than 67% were detected in other plant families (data not shown).

3.3. Evaluation of the resistance to proteolysis of Jun o 4 and Phl p 7

The resistance to simulated gastric and intestinal digestion of rJun o 4 and rPhl p 7 was investigated by treating the two proteins with SGF and SIF, as described in the Materials and Methods section. Fig. 3 (panels A and D) shows that the untreated proteins were eluted as entire molecules showing each one a single sharp peak by RP-HPLC. In contrast, the treatment with the gastric protease pepsin (Fig. 3, panels B and E) or with the intestinal proteases trypsin and chymotrypsin (Fig. 3, panels C and F) caused the fragmentation of both the proteins. In fact, after 120 min of incubation with the gastrointestinal proteases, both the proteins were eluted by RP-HPLC as a mixture of small fragments. These results indicate that both, Jun o 4 and Phl p 7, are degraded by the gastrointestinal proteases.

3.4. IgE binding results and clinical details

8784 consecutive patients reporting allergic symptoms towards any food or inhalant source were analyzed with the FABER test, regardless of their geographic origin or urban/non-urban life context. Therefore, this was considered a random cohort, representing a general population of allergic subjects, since no selection criteria was applied.

Overall, 389 patients (4.4%) proved to be sensitized to Jun o 4 and/or Phl p 7 (Table 3). In particular, out of 8784 tested patients, 70 (0.8%) were IgE positive to both Jun o 4 and Phl p 7, 288 (3.3%) to Jun o 4 only and 31 (0.35%) to Phl p 7 only (Fig. 4).

Among the patients recorded to be sensitized to Jun o 4 and/or Phl p 7, 50 clinical histories were available. They were all Italian patients, living mainly in the Lazio region (Central Italy), reporting the classical symptoms of pollinosis. Rhinitis was the most reported symptom followed by conjunctivitis and asthma (Table 4). Several patients showed multiple pollen sensitization, since they were IgE positive to more than one pollen extract available on the FABER biochip. Table 4 shows the IgE values obtained on the pollen extract of the cypress *Cryptomeria japonica* (Cry j) and *C. sempervirens* (Cup s), of grass *Phleum pratense* (Phl p), *Ambrosia artemisiifolia* (Amb a) and *P. judaica* (Par j) and of the tree *Betula verrucosa* (Bet v) and *O. europaea* (Ole e). Results of IgE binding obtained on two cross-reactive carbohydrate determinants (CCD) markers, a protease of *Ananas comosus* (Ana c 2) and a peroxidase from *Armoracia rusticana* (Arm r HRP), are also shown in Table 4 to provide indications about possible interference of CCD-specific IgE on the data obtained with the extracts. They suggest that the IgE values obtained for some pollen extracts could be affected by the presence of IgE recognizing CCD bound to the proteins contained in the extracts, in about 36% of the patients' sera. Nevertheless, most of the 50 sera do not show IgE specific for the CCD markers, Ana c 2 and Arm r HRP.

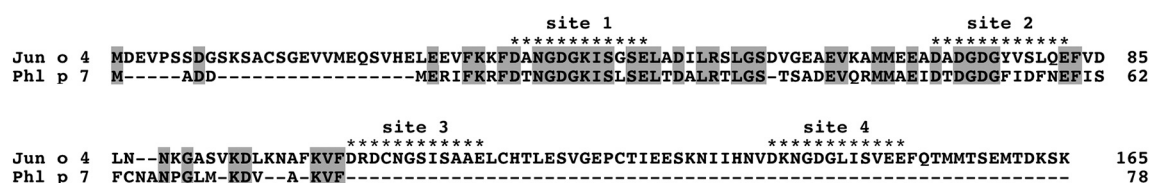


Fig. 2. Amino acid sequence alignment of Jun o 4 and Phl p 7. The single globular domain of Phl p 7 has been aligned with the globular domains at the N-terminal region of Jun o 4. Residues of the calcium-binding sites, comprised in the four EF-hand motifs, are marked with asterisks. Bars indicate gaps that were introduced to align the calcium-binding sites. Residues of Jun o 4 conserved in Phl p 7 are highlighted in grey.

Table 1

Polcalcins showing amino acid sequence identity equal or higher than 67% compared to Jun o 4. Cup a 4 was detected by searching the Allergome database using Jun o 4 as query sequence. Accession numbers are from UniProtKB unless otherwise stated.

Allergen	Accession number	Source	Plant order	Plant family	Number of EF-hand	Identity with Jun o 4 (%)
Jun o 4.0101	O64943	prickly juniper	Cupressales	Cupressaceae	4	100
Cup a 4	A0T2M3	cypress	Cupressales	Cupressaceae	4	90
Cry j polcalcin	GenBank FY282136.1	japanese cedar	Cupressales	Cupressaceae	4	76

Table 2

Polcalcins showing amino acid sequence identity equal or higher than 67% compared to Phl p 7. This list of allergens was obtained by searching the Allergome database using Phl p 7 as query sequence. When more than one protein was detected in an individual plant source that showing the highest sequence identity with Phl p 7 was selected and included in the Table. Accession numbers are from UniProtKB.

Allergen	Accession number	Source	Plant order	Plant family	Number of EF-hand	Identity with Phl p 7 (%)
Phl p 7.0101	O82040	timothy grass	Poales	Poaceae	2	100
Ory s 7	Q6YYX3	rice	Poales	Poaceae	2	100
Cyn d 7	A28050	bermuda grass	Poales	Poaceae	2	97
Bra di 7	I1IM99	purple false brome grass	Poales	Poaceae	2	97
Hor v 7	F2EKW4	barley	Poales	Poaceae	2	97
Sor b 7	C5YN56	sorghum	Poales	Poaceae	2	96
Zea m 7	B6T167	maize	Poales	Poaceae	2	94
Sal k 7.0101	A0A0M3TIL4	prickly saltwort	Caryophyllales	Chenopodiaceae	2	76
Che a 3	Q84V36	goosefoot	Caryophyllales	Chenopodiaceae	2	76
Par j 4.0101	B5QST3	pellitory	Rosales	Urticaceae	2	70
Syr v 3.0101	P58171	lilac	Lamiales	Oleaceae	2	70
Bra n 4	P69196	rape	Brassicales	Brassicaceae	2	69
Bra r 5.0101	P69197	rape	Brassicales	Brassicaceae	2	69
Ole e 3.0101	Q5DTB7	olive tree	Lamiales	Oleaceae	2	69
Nic t 3	Q8VWY6	tobacco	Solanales	Solanaceae	2	68
Aln g 4.0101	O81701	alder	Fagales	Betulaceae	2	68
Bet v 4.0101	Q39419	birch	Fagales	Betulaceae	2	67

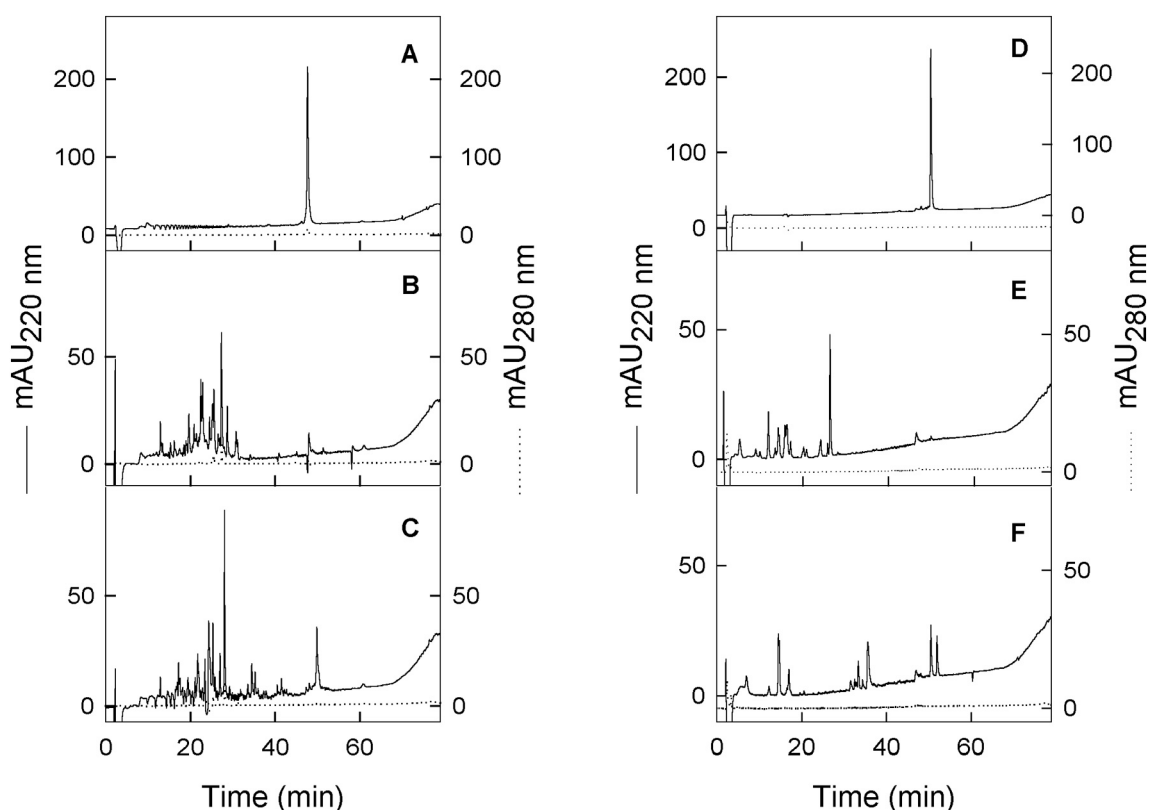


Fig. 3. Digestion stability of Jun o 4 (left panels) and Phl p 7 (right panels). RP-HPLC profiles show untreated proteins (A and D), incubation in SGF for 2 h (B and E) and incubation in SIF for 2 h (C and F).

Table 3

Details of 389 patients sensitized to Jun o 4 and/or Phl p 7 recorded in a random population of 8784 Italian suspected allergics analyzed with the FABER test.

Feature analyzed	Jun o 4 ⁺	Jun o 4 ⁺ Phl p 7 ⁺	Phl p 7 ⁺
Positive sera	288	70	31
Average age (yrs)	24.95	33.63	37.01
Range of age (yrs)	0.56–85.25	2.55–79.66	11.47–73.92
Female	144	32	15
Male	144	38	16

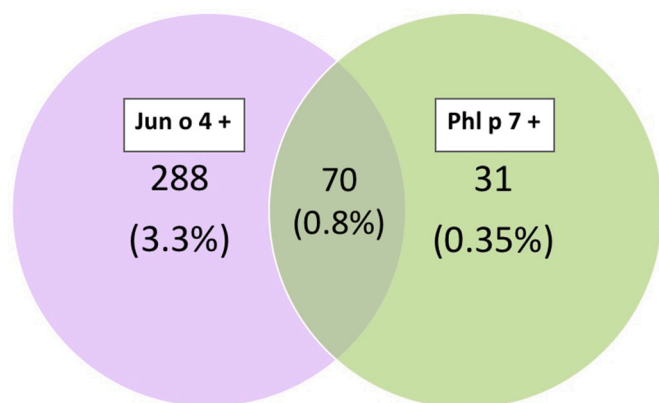


Fig. 4. Venn diagram representing the three cohorts of patients IgE positive to Jun o 4 and/or Phl p 7 identified in a general population of 8784 suspected allergics.

Almost all 50 patients were IgE positive to Cupressaceae total extracts and/or to individual cypress allergenic proteins, such as Jun o 4 (Table 4). On the basis of the recorded IgE positive results, the 50 patients were splitted in four groups (Table 4 and Fig. 5). Eleven patients sensitized to Jun o 4, to Phl p 7 and to the *P. pratense* extract were included in the Group 1; 7 patients IgE negative to Jun o 4, but positive to Phl p 7 and to the *P. pratense* extract in the Group 2; 22 patients sensitized to Jun o 4 and to the *P. pratense* extract, but negative to Phl p 7, in Group 3; 10 patients IgE positive to Jun o 4, but negative to Phl p 7 and to the *P. pratense* extract, in the Group 4. It can be observed that the Groups 1 and 2, and to a lesser extent the Group 3, include patients polysensitized to several pollens, whereas the Group 4 consists of individuals sensitized to cypress pollen, with the only exception of two subjects showing one additional IgE positivity towards the extract of *P. judaica*. These considerations remain still valid even if we take into account the sera containing CCD-specific IgE, that is those positive to Ana c 2 and Arm r HRP.

4. Discussion

Many allergens have been identified in Cupressaceae species. Some of them have been widely characterized, whereas others are still waiting for further investigations. The list of those less characterized includes lipid transfer protein, profilin, chitinase, aspartic protease, subtilisin-like serine protease and isoflavone reductase. The most recent cypress allergen to be identified is Cup s 7 [21]. It was isolated from the *C. sempervirens* pollen. Cup s 7 belongs to the family of gibberellin-regulated proteins (GRP). GRP is a widespread protein and may represent a new panallergen. Allergens considered typical of Cupressaceae pollens have been classified in four groups [13]. Allergens belonging to groups 1 and 2 are pectate lyase and polygalacturonase enzymes, respectively. They both are considered major allergens sensitizing more than 80% of cypress pollen allergic patients. Cypress thaumatin-like proteins are classified as group 3 and are reported as borderline between major and minor allergens. Allergenic polcalcins represent the

group 4 of cypress allergens and they are reported as minor allergens [16]. Nevertheless, Cupressaceae polcalcins have been poorly investigated so far and more studies are required to define their relevance in cypress allergy. Allergenic polcalcins from Cupressaceae so far described include Jun o 4 from prickly juniper and Cup a 4 from cypress [16]. Jun o 4 only is referenced in WHO/IUIS.

The recombinant prickly juniper polcalcin, rJun o 4, here described, was included in a multiplex diagnostic system where it was recognized and bound by specific IgE contained in the sera of pollen allergic patients. Any comparison with the natural allergen was impaired because the protein isolated from the natural source was not available. Experimental results were compared with those obtained for rPhl p 7, the timothy grass polcalcin representing one of the best-characterized allergens of this CBP sub-family. The frequency of IgE reactivity towards individual allergenic polcalcins from various trees, grasses, and weeds was reported to depend on the specific molecule but also on the geographical area, level and time of pollen exposure of the tested patients [28], as well as on the criteria used to select the populations under investigation. In fact, the variability of the frequency of sensitization to specific allergens as a function of specific analyzed populations has been widely described. For instance, a prevalence of reactivity to the birch pollen Bet v 4 between 5% and 11% was reported for a population of pollen allergics from North and Central Europe, whereas 27% was observed for Italian patients [29]. A prevalence of 46% was described for Che a 3 in a goosefoot pollen allergic population [30]. A prevalence of 9.6% sera IgE positive to Cup a 4 was detected in a cohort of 177 patients sensitized to *C. arizonica* pollen [16]. A frequency of sensitization to Bet v 4 corresponding to 23% was reported in a cohort of pollen allergic patients from a birch-free area [31]. The same percentage of IgE positive sera (8.9%) was recorded for Phl p 7 and Bet v 4 when a population of 112 pollen allergic patients suffering from rhinitis was analyzed [32]. A prevalence of sensitization to Phl p 7 between 7 and 10% was described in a cohort of grass allergic subjects [6,33]. It is worth to note that these percentages were registered in cohorts of selected allergics where the features of patients and the calculated prevalences depended on the criteria used for the enrolment. In order to avoid as much as possible this kind of bias, we analyzed the results obtained from a set of subsequent patients tested with the FABER test without the application of any selection.

The study here reported provides new information about the patterns of sensitization to allergenic polcalcins in a general population of allergic subjects. In contrast with previous studies [15,17], which found the sensitization to Jun o 4 always associated with that to Phl p 7, the large population of 8784 subjects here analyzed allowed the detection of a noticeable cohort of pollen allergic patients (3.3%) sensitized to the cypress polcalcin only. An in depth analysis of a representative group of 50 Italian patients suffering from pollinosis, and sensitized to Jun o 4 and/or Phl p 7, showed that rhinitis was the most prevalent reported symptom. Lower frequencies of other symptoms, such as conjunctivitis and asthma, were observed in all the analyzed patients, independently of the sensitization to Jun o 4 and/or Phl p 7. The analysis of the sensitization data of the cohort of 50 Italian patients has driven the fractionation of this cohort in four groups with specific patterns of sensitization. Group 1 represents a sub-population with a pattern of sensitization already reported in a previous study [17] and showing that no patient was sensitized to rJun o 4 only. In fact, it included patients co-sensitized to both Jun o 4 and Phl p 7. However, in that study [17] the authors analyzed a selected population of grasses allergic patients speculating that polcalcins with 2EF-hand (in that study they were rPhl p 7 and rAln g 4) either cause more frequent sensitization than the 4EF-hand allergens, such as Jun o 4, or that they contain more cross-reactive IgE epitopes. Group 2 includes Phl p 7+ Jun o 4- patients that, like those of Group 1, show sensitization to some other pollens from different sources, such as Cupressaceae, grass and tree other than Cupressaceae. These data are in line with literature reporting that the sensitization to Phl p 7 is more frequent in polysensitized patients [34]. It is worth noting

Table 4

Immunological and clinical data of 50 Italian patients IgE positive to Jun o 4 and/or Phl p 7 for which a clinical history was available.

Patients details			Symptoms ^a			Faber 244 (FIU) ^b												
Groups	Sex	Age	R	C	A	Jun o 4	Phl p 7	Phl p	Cry j	Cup s	Amb a	Bet v	Ole e	Par j	Ana c 2	Arm r HRP		
Group 1	1	F	46	1	1	1	12.42	9.25	10.45	0.65	0	0.91	0	1.08	33.04	0	0	
	2	F	77	1	1	1	6.95	1.44	6.03	2.41	0	2.88	3.34	2.89	14.61	0	0	
	3	F	60	1	1	1	21.61	34.66	89.8	3.42	5.79	3.34	5.58	12.39	46.02	3.08	0.25	
	4	M	27	0	1	1	138.58	128.29	151.82	12.46	12.48	9.43	16.24	69.74	125.78	4.91	7.96	
	5	M	13	1	0	1	12.32	113.19	151.65	23.26	11.12	7.26	19.47	119.09	100.4	7.32	6.94	
	Jun o 4 ⁺	6	F	50	1	1	1	2.68	15.06	32.75	1.34	2.89	2.8	5.52	27.7	26.42	0.22	3.99
	Phl p 7 ⁺	7	F	25	0	1	1	21.95	8.8	57.41	1.81	1.36	0	1.36	5.43	17.14	0	0
	Phl p ⁺	8	F	44	0	0	1	20.24	30.35	34.89	0	0	1.63	0	32.37	93.12	0	0
		9	F	41	1	1	1	4.05	1.99	70.56	4.16	0	0.93	1.63	6.64	76.24	0	7.08
		10	M	22	1	0	0	0.24	1.21	37.49	0.73	1.68	0	0	1.45	3.38	0	0
Group 2	11	M	21	0	0	1	0.23	0.23	147.2	15.94	14.23	11.36	4.38	13.18	105.9	3.54	3.43	
	12	F	50	1	1	1	0	1.24	4.98	0	11.11	0	2.48	22.15	3.73	1.24	0	
	13	F	32	1	0	1	0	1.55	2.58	0	0	0	3.08	2.07	0	0	0	
	Jun o 4 ⁺	14	F	36	1	0	1	0	111.26	86.18	0	6.88	5.96	18.18	67.97	132.7	4.95	5.95
	Phl p 7 ⁺	15	M	35	1	1	1	0	24.63	168.85	2.52	0	3.52	6.25	28.35	30.26	1.12	0
	Phl p ⁺	16	M	33	0	0	1	0	2.16	0.81	0.46	3.29	0	0	1.37	10.57	0	0
		17	M	17	1	1	0	0	2.96	39.19	19.37	37.23	9.74	4.88	11.3	9.77	13.65	0
		18	F	47	1	1	0	0	2.98	7.73	10.3	0	2.13	1.17	5.19	32.24	2.23	0
		19	F	14	1	0	0	2.3	0	4.86	0	0	0	0	0	0	0	0
		20	F	66	1	0	0	0.71	0	0.71	0	0.24	0	0	3.98	0	0	0
Group 3	21	F	18	1	1	0	1.42	0	4.45	0	2.32	0	0	0	0	0	0	
	22	F	19	1	1	0	1.18	0	8.5	4.27	4.29	0	0	6.21	0	0	0	
	23	F	15	1	1	0	0.71	0	105.2	4.19	5.75	10.38	6.59	32.24	3.85	12.91	0	
	24	F	19	1	1	1	6.58	0	21.37	0	2.19	0	0	0	2.79	0	0	
	25	F	35	0	0	1	1.74	0	2.08	0	2.08	0	1.55	1.75	0.7	0	0	
	26	F	23	1	0	1	0.52	0	1.18	0	0.52	0	0	0	0	0	0	
	27	F	31	1	1	1	0.46	0	4.65	0.69	1.94	0	0	0	0	0	0	
	Jun o 4 ⁺	28	M	39	1	0	1	0.53	0	2.12	0.92	2.78	0	0	0	11	0	0
	Phl p 7 ⁺	29	F	27	0	0	1	0.46	0	0.7	0.46	6.81	0.83	1.39	0	0.92	0	0
	Phl p ⁺	30	M	30	0	1	1	0.52	0	15.8	1.05	4.3	0	0	3.14	0	0	0
Group 4	31	F	44	1	1	1	0.51	0	1.02	1.02	3.18	0	0	0	0	0	0	
	32	M	19	0	1	1	0.75	0	1.27	0	1.77	0	0	3.44	0	0	0	
	33	F	24	1	1	1	0.46	0	0.92	0	0	0	1.38	0	0	0	1.37	
	34	F	26	0	1	1	0.41	0	59.04	3.96	0	0	4.2	4.99	2.78	2.39	1.59	
	35	M	46	1	1	1	9.32	0	129.98	10.1	10.76	1.04	13.84	2.6	123.25	0.79	6.02	
	36	M	19	1	0	1	0.58	0	2.92	0	7.75	0	0	13.12	0	0	0	
	37	F	40	1	0	0	2.75	0	12.93	1.82	35.37	17.16	0.92	34.73	49.28	5.89	4.36	
	38	M	12	1	0	1	0.9	0	5.26	0	2.26	4.02	8.83	53.09	3.87	0.3	2.39	
	39	M	35	1	1	1	2.96	0	132.39	87.89	78.82	110.08	67.24	113.12	101.1	113.66	102.85	
	40	M	39	1	0	0	1.08	0	2.16	0	0.72	0	0	0	11.97	0	0	

Phl p, Cry j, Cup s, Amb a, Bet v, Ole e and Par j indicate the extracts of *Phleum pratense*, *Cryptomeria japonica*, *Cupressus sempervirens*, *Ambrosia artemisiifolia*, *Betula verrucosa*, *Olea europea* and *Parietaria judaica*

^a R, rhinitis; C, conjunctivitis; A, asrhama.

^b FIU, FABER International Units, positive FIU ≥ 0.01 .

that, in the present study, a sub-population (Group 4) displaying sensitizations limited to Jun o 4 and to some Cupressaceae pollens has been detected. The Group 4 is also characterized by a negative reaction towards CCD markers, thus excluding any interference of CCD in the obtained results. Therefore, it appears that the sensitization to Jun o 4 can be found isolated from that to Phl p 7 and to a broad panel of pollens from different taxonomic groups. The sensitization pattern of Group 4 had never been reported before, probably due to the criteria applied for the selection of the population under investigation. In this study, with the aim to analyze a general population, no selection criteria were applied, thus allowing the detection of the Group 4 of pollen sensitized patients, and contributing to increase the knowledge on the patterns of sensitization to polcalcins. Definitely, the data obtained highlight that Phl p 7 cannot be considered a marker of sensitization towards all the

polcalcins, and that the use of a Cupressaceae polcalcin is needed to improve the diagnosis of allergy towards cypress pollen.

Some allergens are considered pollen-specific and therefore lacking homologs that could cause cross-reactions in plant foods. Probably for this reason, their resistance to gastrointestinal digestion has not received attention. However, pollens constantly come in contact not only with respiratory mucosa but also with the oral mucosa causing irritation, itching and inflammation of the mouth and the pharynx [35]. Then, after swallowing the pollen can move forward along the gastrointestinal tract. In addition to the inhalation, pollen can be introduced in the gastrointestinal tract with the consumption of honey and of some dietary supplements, such as bee-pollen [36,37]. Polcalcins are released from the pollen grain during contact with the mucosa [6] and therefore they could contribute to the allergic reactions involving the gastrointestinal

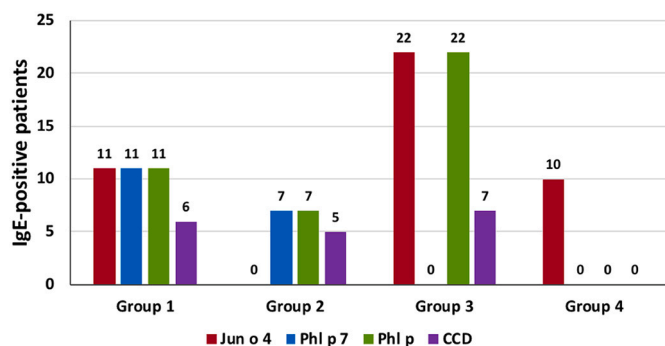


Fig. 5. Patients IgE positive to Jun o 4 and/or Phl p 7 and/or Phl p extract. Based on the sensitization recorded, the cohort of 50 Italian patients suffering from pollinosis was splitted in 4 groups. Additional details are reported in Table 4.

tract. In this context, the knowledge of the sensitivity to gastrointestinal digestion of polcalcins can help the understanding of their role in the induction of allergic symptoms observed in sensitized patients. The experimental results here reported indicate that Jun o 4 and Phl p 7 are not stable to the gastric and intestinal digestion, which cause fragmentation of the proteins. This feature is in common with other pollen allergens, such as Bet v 1-related proteins and profilins, which cause the classical symptoms of OAS associated to pollinosis [38]. The degradation of the two analyzed polcalcins due to gastrointestinal proteases suggests that, similar to Bet v 1-like allergens and profilins, they should not be responsible of severe symptoms, such as nausea, diarrhoea, vomiting and anaphylaxis, when ingested inside the pollen or honey [39,40]. In fact, these symptoms are generally assigned to allergens stable to gastrointestinal digestion. Conversely, given their low stability, it can be hypothesized that ingested polcalcins could contribute to cause mild symptoms localized in the mouth especially in periods of intense pollination in which many patients report itching in the throat in addition to rhinitis. However, these results can be considered preliminary to further studies needed to verify the above hypothesis, as well as to assess the allergenicity of protein fragments derived from gastrointestinal digestion.

Literature reports also suggest that profilins and Bet v 1-related proteins may trigger eosinophilic esophagitis (EoE) in a subset of pollen allergic patients [41]. Sensitization to some polcalcins was also reported in adult patients with EoE [42]. Recently, cypress pollinosis has been associated to a major incidence of EoE [43]. Further studies are needed to understand the role of polcalcins in the EoE observed in patients suffering from cypress pollinosis. Actually, in depth investigations are claimed to understand the clinical relevance of polcalcins in the respiratory and gastrointestinal allergy diseases, which is still debated, as well as their role as possible cause of side effects in the oral immunotherapy for pollinosis [44].

In conclusion, the recombinant Jun o 4 here described proved to be suitable for the detection of specific IgE in the sera of Cupressaceae allergic patients. In addition, the pattern of sensitization of Jun o 4 demonstrates the need to include this allergen in the diagnostic tests. Therefore, this molecule can be included in allergy diagnostic systems and contribute to detect the sensitization towards cypress pollen. In addition, this study confirms literature reporting that patients sensitized to the grass polcalcin Phl p 7 are generally grouped in cohorts characterized by multiple pollen (from grasses and trees) sensitization, but it highlights that the same is not true for the cypress polcalcin. In fact, patients sensitized to Jun o 4 can be splitted partially in a cohort with multiple pollen sensitization (like Phl p 7) and partially in a second cohort showing sensitization limited to cypress pollen. This means that the grass polcalcin, Phl p 7, cannot be further considered a marker of sensitization towards all the polcalcins. Conversely, a cypress polcalcin, like Jun o 4, is needed to improve the diagnosis of allergy towards

Cupressaceae pollen. A future exploitation of Jun o 4 in sensitization and allergy diagnosis, combined with investigation of symptoms at the nasal and gastrointestinal level, can contribute to understand the clinical relevance of polcalcins in general, and in particular the relevance of cypress polcalcin.

Author contributions

Study planning, MAC, CA, MT and AM; production of recombinant allergen and its characterization, TR, IG, AFD and LT; immunochemical experiments, CR; data analysis, writing, review and editing, TR, IG, CA, MT and MAC. All authors have read and agreed to this version of the manuscript.

Funding

The authors would like to acknowledge the International Fund for Advanced Research in Allergy and Immunology (IFARAI) Onlus (Italy) for supporting this research. The study was also partially funded by ADL S.r.l. R&D internal funding.

Declaration of Competing Interest

CA and AM as ADL stakeholders, develop, manufacture and distribute the FABER test as a diagnostic service. CR as employee of CAAM, TR, IG and LT as employees of ADL, contribute to develop, manufacture and perform the FABER test. MAC and MT receive funding from ADL.

Acknowledgments

We thank Dr. Immacolata Fiume for her kind assistance with the amino acid sequencing experiments and Dr. Giovanni del Monaco for his technical assistance.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.clim.2021.108894>.

References

- [1] I. Pablos, M. Egger, E. Vejvar, V. Reichl, P. Briza, D. Zennaro, C. Rafaiani, W. Pickl, B. Bohle, A. Mari, et al., Similar Allergenicity to different *Artemisia* species is a consequence of highly cross-reactive art v 1-like molecules, *Medicina* (Kaunas, Lithuania) 55 (8) (2019) 504.
- [2] A. Barre, H. Benoist, P. Rougé, Allergènes moléculaires des pollens : où en sommes-nous ? *Rev. Française d'Allergol.* 59 (8) (2019) 592–604.
- [3] N. Wopfner, O. Dissertori, F. Ferreira, P. Lackner, Calcium-binding proteins and their role in allergic diseases, *Immunol. Allergy Clin. N. Am.* 27 (1) (2007) 29–44.
- [4] H. Kawasaki, R.H. Kretsinger, Calcium-binding proteins. 1: EF-hands, *Protein Profile* 1 (4) (1994) 343–517.
- [5] M. Raith, D. Zach, L. Sonnleitner, K. Woroszylo, M. Focke-Tejkl, H. Wank, T. Graf, A. Kuehn, M. Pascal, R.M. Muñoz-Cano, et al., Rational design of a hypoallergenic Phl p 7 variant for immunotherapy of polcalcin-sensitized patients, *Sci. Rep.* 9 (1) (2019) 7802.
- [6] V. Niederberger, B. Hayek, S. Vrtala, S. Laffer, A. Twardosz, L. Vangelista, W. R. Sperr, P. Valent, H. Rumpold, D. Kraft, et al., Calcium-dependent immunoglobulin E recognition of the apo- and calcium-bound form of a cross-reactive two EF-hand timothy grass pollen allergen, Phl p 7, *FASEB J.* 13 (8) (1999) 843–856.
- [7] E. Engel, K. Richter, G. Obermeyer, P. Briza, A.J. Kungl, B. Simon, M. Auer, C. Ebner, H.J. Rheinberger, M. Breitenbach, et al., Immunological and biological properties of Bet v 4, a novel birch pollen allergen with two EF-hand calcium-binding domains, *J. Biol. Chem.* 272 (45) (1997) 28630–28637.
- [8] R. Asero, L. Jimeno, D. Barber, Preliminary results of a skin prick test-based study of the prevalence and clinical impact of hypersensitivity to pollen panallergens (polcalcin and profilin), *J. Investig. Allergol. Clin. Immunol.* 20 (1) (2010) 35–38.
- [9] K. Toriyama, T. Okada, M. Watanabe, T. Ide, T. Ashida, H. Xu, M.B. Singh, A cDNA clone encoding an IgE-binding protein from *Brassica* anther has significant sequence similarity to Ca(2+)-binding proteins, *Plant Mol. Biol.* 29 (6) (1995) 1157–1165.

- [10] K.S. Rathore, R.J. Cork, K.R. Robinson, A cytoplasmic gradient of Ca²⁺ is correlated with the growth of lily pollen tubes, *Dev. Biol.* 148 (2) (1991) 612–619.
- [11] C.S. Bokka, G.K. Veeramachaneni, V. Thunuguntla, N.K. Manda, J.S. Bondili, Specific panallergen peptide of Sorghum Polcalcin showing IgE response identified based on in silico and in vivo peptide mapping, *Biosci. Rep.* 39 (11) (2019).
- [12] A. Ledesma, E. González, C.Y. Pascual, J. Quiralte, M. Villalba, R. Rodríguez, Are Ca²⁺-binding motifs involved in the immunoglobulin E-binding of allergens? Olive pollen allergens as model of study, *Clin. Exp. Allergy* 32 (10) (2002) 1476–1483.
- [13] D. Charpin, C. Pichot, J. Belmonte, J.P. Sutra, J. Zidkova, P. Chanez, Y. Shahali, H. Sénéchal, P. Poncet, Cypress Pollinosis: from tree to clinic, *Clin. Rev. Allergy Immunol.* 56 (2) (2019) 174–195.
- [14] P. Iacovacci, C. Afferni, B. Barletta, R. Tinghino, G. Di Felice, C. Pini, A. Mari, Juniperus oxycedrus: a new allergenic pollen from the Cupressaceae family, *J. Allergy Clin. Immunol.* 101 (6 Pt 1) (1998) 755–761.
- [15] R. Tinghino, B. Barletta, S. Palumbo, C. Afferni, P. Iacovacci, A. Mari, G. Di Felice, C. Pini, Molecular characterization of a cross-reactive Juniperus oxycedrus pollen allergen, Jun o 2: a novel calcium-binding allergen, *J. Allergy Clin. Immunol.* 101 (6 Pt 1) (1998) 772–777.
- [16] Y. Pico de Coaña, N. Parody, M. Fuertes, J. Carnés, D. Roncarolo, R. Ariano, J. Sastre, G. Mistrello, C. Alonso, Molecular cloning and characterization of cup a 4, a new allergen from Cupressus arizonica, *Biochem. Biophys. Res. Commun.* 401 (3) (2010) 451–457.
- [17] R. Tinghino, A. Twardosz, B. Barletta, E.M. Puggioni, P. Iacovacci, C. Butcheroni, C. Afferni, A. Mari, B. Hayek, G. Di Felice, et al., Molecular, structural, and immunologic relationships between different families of recombinant calcium-binding pollen allergens, *J. Allergy Clin. Immunol.* 109 (2) (2002) 314–320.
- [18] M.L. Bernardi, I. Giangrieco, L. Camardella, R. Ferrara, P. Palazzo, M.R. Panico, R. Crescenzo, V. Carratore, D. Zennaro, M. Liso, et al., Allergenic lipid transfer proteins from plant-derived foods do not immunologically and clinically behave homogeneously: the kiwifruit LTP as a model, *PLoS One* 6 (11) (2011), e27856.
- [19] C. Ciacci, I. Russo, C. Bucci, P. Iovino, L. Pellegrini, I. Giangrieco, M. Tamburrini, M.A. Ciardiello, The kiwi fruit peptide kisser displays anti-inflammatory and anti-oxidant effects in in-vitro and ex-vivo human intestinal models, *Clin. Exp. Immunol.* 175 (3) (2014) 476–484.
- [20] L. Tuppo, I. Giangrieco, C. Alessandri, T. Ricciardi, C. Rafeiani, M. Ciancamerla, R. Ferrara, D. Zennaro, M.L. Bernardi, M. Tamburrini, et al., Pomegranate chitinase III: identification of a new allergen and analysis of sensitization patterns to chitinases, *Mol. Immunol.* 103 (2018) 89–95.
- [21] L. Tuppo, C. Alessandri, I. Giangrieco, M. Ciancamerla, C. Rafeiani, M. Tamburrini, M.A. Ciardiello, A. Mari, Isolation of cypress gibberellin-regulated protein: analysis of its structural features and IgE binding competition with homologous allergens, *Mol. Immunol.* 114 (2019) 189–195.
- [22] I. Giangrieco, T. Ricciardi, C. Alessandri, L. Farina, R. Crescenzo, L. Tuppo, M. Ciancamerla, C. Rafeiani, M.L. Bernardi, A.F. Digilio, et al., ENEA, a peach and apricot IgE-binding protein cross-reacting with the latex major allergen Hev b 5, *Mol. Immunol.* 112 (2019) 347–357.
- [23] C. Alessandri, R. Ferrara, M.L. Bernardi, D. Zennaro, L. Tuppo, I. Giangrieco, M. Tamburrini, A. Mari, M.A. Ciardiello, Diagnosing allergic sensitizations in the third millennium: why clinicians should know allergen molecule structures, *Clin. Transl. Allergy* 7 (2017) 21.
- [24] R. D'Avino, M.L. Bernardi, M. Wallner, P. Palazzo, L. Camardella, L. Tuppo, C. Alessandri, H. Breiteneder, F. Ferreira, M.A. Ciardiello, et al., Kiwifruit Act d 11 is the first member of the ripening-related protein family identified as an allergen, *Allergy* 66 (7) (2011) 870–877.
- [25] C. Notredame, D.G. Higgins, J. Heringa, T-Coffee: a novel method for fast and accurate multiple sequence alignment, *J. Mol. Biol.* 302 (1) (2000) 205–217.
- [26] A. Mari, C. Rasi, P. Palazzo, E. Scala, Allergen databases: current status and perspectives, *Curr Allergy Asthma Rep* 9 (5) (2009) 376–383.
- [27] S.K. Chan, A. Pomés, C. Hilger, J.M. Davies, G. Mueller, A. Kuehn, A.L. Lopata, G. Gadermaier, M. van Hage, M. Raulf, et al., Keeping allergen names clear and defined, *Front. Immunol.* 10 (2019) 2600.
- [28] M. Hauser, A. Roulias, F. Ferreira, M. Egger, Panallergens and their impact on the allergic patient, *Allergy, Asthma Clin. Immunol.* 6 (1) (2010) 1.
- [29] R. Movérare, K. Westritschnig, M. Svensson, B. Hayek, M. Bende, G. Pauli, R. Sorva, T. Haahtela, R. Valenta, L. Elfman, Different IgE reactivity profiles in birch pollen-sensitive patients from six European populations revealed by recombinant allergens: an imprint of local sensitization, *Int. Arch. Allergy Immunol.* 128 (4) (2002) 325–335.
- [30] R. Barderas, M. Villalba, C.Y. Pascual, E. Batanero, R. Rodríguez, Profilin (Che a 2) and polcalcin (Che a 3) are relevant allergens of Chenopodium album pollen: isolation, amino acid sequences, and immunologic properties, *J. Allergy Clin. Immunol.* 113 (6) (2004) 1192–1198.
- [31] A. Mari, M. Wallner, F. Ferreira, Fagales pollen sensitization in a birch-free area: a respiratory cohort survey using Fagales pollen extracts and birch recombinant allergens (rBet v 1, rBet v 2, rBet v 4), *Clin. Exp. Allergy* 33 (10) (2003) 1419–1428.
- [32] K. Kowal, A. Pampuch, E. Sacharzewska, E. Swiebocka, Z. Siergiejko, G. Siergiejko, M. Chruszcz, L. DuBuske, Serum immunoglobulin E reactivity to cross-reacting panallergen components in north-eastern Poland patients pollen sensitized, *Allergy Asthma Proc.* 41 (3) (2020) 183–191.
- [33] A. Mari, Skin test with a timothy grass (Phleum pratense) pollen extract vs. IgE to a timothy extract vs. IgE to rPhl p 1, rPhl p 2, nPhl p 4, rPhl p 5, rPhl p 6, rPhl p 7, rPhl p 11, and rPhl p 12: epidemiological and diagnostic data, *Clin. Exp. Allergy* 33 (1) (2003) 43–51.
- [34] L. Kazemi-Shirazi, V. Niederberger, B. Linhart, J. Lidholm, D. Kraft, R. Valenta, Recombinant marker allergens: diagnostic gatekeepers for the treatment of allergy, *Int. Arch. Allergy Immunol.* 127 (4) (2002) 259–268.
- [35] A. Armentia, S. Martín-Armentia, R. Álvarez-Nogal, B.M. Armentia, M.J. Gayoso, D. Fernández-González, Germination of pollen grains in the oesophagus of individuals with eosinophilic oesophagitis, *Clin. Exp. Allergy* 49 (4) (2019) 471–473.
- [36] F. Paola, D.D. Pantalea, C. Gianfranco, F. Antonio, V. Angelo, N. Eustachio, D. L. Elisabetta, Oral allergy syndrome in a child provoked by royal jelly, *Case Rep. Med.* 2014 (2014), 941248.
- [37] L. Bauer, A. Kohlich, R. Hirschwehr, U. Siemann, H. Ebner, O. Scheiner, D. Kraft, C. Ebner, Food allergy to honey: pollen or bee products? Characterization of allergenic proteins in honey by means of immunoblotting, *J. Allergy Clin. Immunol.* 97 (1 Pt 1) (1996) 65–73.
- [38] C. Alessandri, R. Ferrara, M.L. Bernardi, D. Zennaro, L. Tuppo, I. Giangrieco, T. Ricciardi, M. Tamburrini, M.A. Ciardiello, A. Mari, Molecular approach to a patient's tailored diagnosis of the oral allergy syndrome, *Clin. Transl. Allergy* 10 (2020) 22.
- [39] N. Fuiano, C. Incorvaia, G.G. Riario-Sforza, G. Casino, Anaphylaxis to honey in pollinosis to mugwort: a case report, *Eur Ann Allergy Clin Immunol* 38 (10) (2006) 364–365.
- [40] M. Katayama, M. Aoki, S. Kawana, Case of anaphylaxis caused by ingestion of royal jelly, *J. Dermatol.* 35 (4) (2008) 222–224.
- [41] M. Egan, D. Atkins, What is the relationship between eosinophilic esophagitis (EoE) and aeroallergens? Implications for allergen immunotherapy, *Curr Allergy Asthma Rep* 18 (8) (2018) 43.
- [42] A. Armentia, S. Martín, J. Barrio, B. Martín, J.C. García, J.M. Vega, A. Sánchez, P. Fernández, A. Corell, Value of microarray allergen assay in the management of eosinophilic oesophagitis, *Allergol Immunopathol (Madr)* 43 (1) (2015) 73–80.
- [43] Izquierdo E. La Orden, I. Mahillo-Fernández, S. Fernández Fernández, J. Barrio Torres, E. Román Riechmann, C. Gutiérrez Junquera, Madrid WgoEotGgi: rising trend in pediatric eosinophilic esophagitis incidence in Spain: results of a prospective study 2014–16, *Pediatr. Allergy Immunol.* 32 (6) (2021) 1307–1315.
- [44] S. Miehleke, O. Alpan, S. Schröder, A. Straumann, Induction of eosinophilic esophagitis by sublingual pollen immunotherapy, *Case Rep. Gastroenterol.* 7 (3) (2013) 363–368.

SUPPLEMENTARY INFORMATION

ATG	CGG	GGT	TCT	CAT	CAT	CAT	CAT	CAT	CAT	GGT	ATG	GCT	AGC	ATG	45
M	R	G	S	H	H	H	H	H	H	G	M	A	S	M	15
ACT	GGT	GGA	CAG	CAA	ATG	GGT	CGG	GAT	CTG	TAC	GAC	GAT	GAC	GAT	90
T	G	G	Q	Q	M	G	R	D	L	Y	D	D	D	D	30
AAG	GAT	CGA	TGG	GGA	TCC	atg	gac	gaa	gtt	ccg	tca	agt	gac	gga	135
K	D	R	W	G	S	M	D	E	V	P	S	S	D	G	45
tct	aag	tcg	gca	tgc	tct	ggc	gaa	gtg	gtt	atg	gaa	cag	agc	gtt	180
S	K	S	A	C	S	G	E	V	V	M	E	Q	S	V	60
cac	gaa	ttg	gaa	gag	gtt	ttc	aag	aaa	ttt	gat	gcg	aac	ggg	gac	225
H	E	L	E	E	V	F	K	K	F	D	A	N	G	D	75
gga	aag	atc	tca	gga	tca	gag	ctt	gca	gac	atc	ttg	cgg	tcg	ctg	270
G	K	I	S	G	S	E	L	A	D	I	L	R	S	L	90
gga	agt	gat	gta	ggc	gag	gca	gag	gtc	aag	gcc	atg	atg	gag	gag	315
G	S	D	V	G	E	A	E	V	K	A	M	M	E	E	105
gct	gac	gcg	gac	ggt	gac	ggt	tat	gtt	agc	ctg	caa	gag	ttt	gtg	360
A	D	A	D	G	D	G	Y	V	S	L	Q	E	F	V	120
gat	ctg	aat	aat	aaa	ggt	gct	tct	gtg	aag	gat	ttg	aaa	aat	gct	405
D	L	N	N	K	G	A	S	V	K	D	L	K	N	A	135
ttc	aaa	gtg	ttt	gat	cgg	gac	tgt	aat	ggc	tcc	att	tca	gct	gct	450
F	K	V	F	D	R	D	C	N	G	S	I	S	A	A	150
gag	ctg	tgc	cat	act	ctc	gaa	agc	gtg	ggc	gag	ccc	tgc	acc	atc	495
E	L	C	H	T	L	E	S	V	G	E	P	C	T	I	165
gag	gag	tct	aag	aac	att	att	cac	aac	gtc	gac	aaa	aat	ggg	gat	540
E	E	S	K	N	I	I	H	N	V	D	K	N	G	D	180
gga	ctt	att	agt	gtt	gaa	gaa	ttt	cag	aca	atg	atg	aca	agt	gaa	585
G	L	I	S	V	E	E	F	Q	T	M	M	T	S	E	195
atg	act	gat	aag	agc	aaa	tga	tag								609
M	T	D	K	S	K	*	*								201

Supplementary Figure 1. Nucleotide sequence and encoded amino acids of recombinant Jun o 4 (rJun o 4). rJun o 4 contains 165 amino acids (in bold), plus the 36 residues at the N-terminus containing the 6xHis tag.