

The natural profilin from Russian thistle (*Salsola kali*) contains a low IgE-binding ability isoform – molecular and immunological characterization

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Chenopodiaceae pollens such as those from *Salsola kali* and *Chenopodium album* are important causes of allergy in Mediterranean areas because of the progress of desertification in European countries. Among the various allergenic protein families, profilins constitute a group of pan-allergens that are involved in polysensitization and pollen-food allergy syndrome. Two-dimensional electrophoresis analysis of *S. kali* profilin highlighted a polymorphic pattern, with several isoforms that have different molecular features (isoelectric point and molecular mass) and immunological features. Two isoforms have been cloned and sequenced. Sal k 4.02 and Sal k 4.03 displayed non-conservative amino acid changes in critical positions of the IgE epitopes. Both isoforms were produced in *Escherichia coli* and structurally and spectroscopically characterized. Changes in the electrophoretic mobility and in their IgG and IgE immunological behavior were observed in comparison with Che a 2, their counterpart from *C. album*. The IgE-binding ability of Sal k 4.03 is similar to that of Che a 2, whereas Sal k 4.02 showed a 35% reduction in IgE binding in 86% of patients, suggesting a hypoallergenic character. Three-dimensional modeling allowed us to propose which amino acid residues are involved in those immunological changes based on epitope mapping studies previously performed in other profilins. These profilin isoforms constitute suitable candidates for specific immunotherapy with recombinant allergens.

Introduction

Type I allergy is a generalized health problem in industrialized countries, affecting ~20–25% of the general population [1,2]. Among the most frequent allergies, those induced by pollens, commonly named pollinosis, are one of the most widespread [3]. In this immunological disorder, whose prevalence has significantly increased in the last years, overproduction of IgE antibodies is the starting point triggering symptoms such as rhinitis, conjunctivitis or asthma [4].

Russian thistle (*Salsola kali*) belongs to the Chenopodiaceae family and constitutes a source of allergenic pollen. The ubiquitous distribution of this plant in temperate areas such as middle eastern countries, the USA and southern Europe means that this weed has become an important allergy-inducing agent in recent years [5,6]. Moreover, the desertification process observed in some regions, its use in greening programs and the pollution derived from oil industries have increased the presence of *S. kali* in certain areas. This

weed produces large amounts of easily propagated anemophilous pollen that is responsible for a high incidence of allergy.

Specific allergenic markers, such as Sal k 1, a pectin methyl esterase considered to be the major allergen of *S. kali* pollen [7], and polysensitization markers such as profilins from various Chenopodiaceae species have been identified in the allergen profile of pollen of these species [8]. Profilins are small proteins (12–15 kDa) that are found in all eukaryotic cells. They stimulate ADP/ATP turnover in actin filament assembly, and are implicated in cellular signaling pathways through phosphatidylinositol phosphates [9]. Plant profilins have been shown to be allergens in pollen from *Chenopodium album*, *Betula verrucosa*, *Phleum pratense*, *Phoenix canariensis*, etc. [10–13], foods such as *Cucumis melo*, *Citrullus lanatus*, *Arachis hypogae*, etc. [14–16] or in latex from *Hevea brasiliensis* [17]. Profilins are proteins with high conserved amino acid sequences, with usually more than 75% identity between species, and they are pan-allergens due to marked IgE cross-reactivity among species [18,19]. Profilins are mainly involved in airway, latex or food sensitization cross-reactivities. Although previous studies have shown that various profilins from plant sources bind IgE from profilin-sensitized patients with similar affinity [20], the presence of particular isoforms with low IgE-binding capability in the natural sources cannot be discounted. These may be useful tools to identify the critical amino acids involved in IgE binding and to establish specific allergen epitope therapies.

Due to the low amounts of profilin in *S. kali* pollen, its recombinant production at high yields is essential for clinical and biochemical purposes. The availability of such recombinant proteins allows use of standardized diagnostic protocols in arrays of hundreds of proteins, and certain techniques may be applied to modify their allergenicity to obtain hypoallergenic variants [21–23]. In addition to classical treatments for allergy such as antihistamines or immunosuppressive drugs, immunomodulatory therapy may be performed using such modified allergens [22,24].

In the study presented here, we have cloned, sequenced and produced two profilin isoforms from *S. kali* (Sal k 4.02 and Sal k 4.03) in *Escherichia coli* in order to study their immunological and antigenic properties. The isoforms exhibited different IgE-binding capacity, and identified the amino acid changes responsible for these differences. According to the immunological data obtained, the natural pollen profilin Sal k 4.02 has reduced IgE-binding ability, suggesting its potential utilization in desensitization protocols.

Results

Identification of the profilin allergen in *S. kali* pollen extract by 2D electrophoresis

The protein content of *S. kali* pollen extract was analyzed by 2D electrophoresis after silver staining (Fig. 1A). Profilin was detected using an anti-profilin

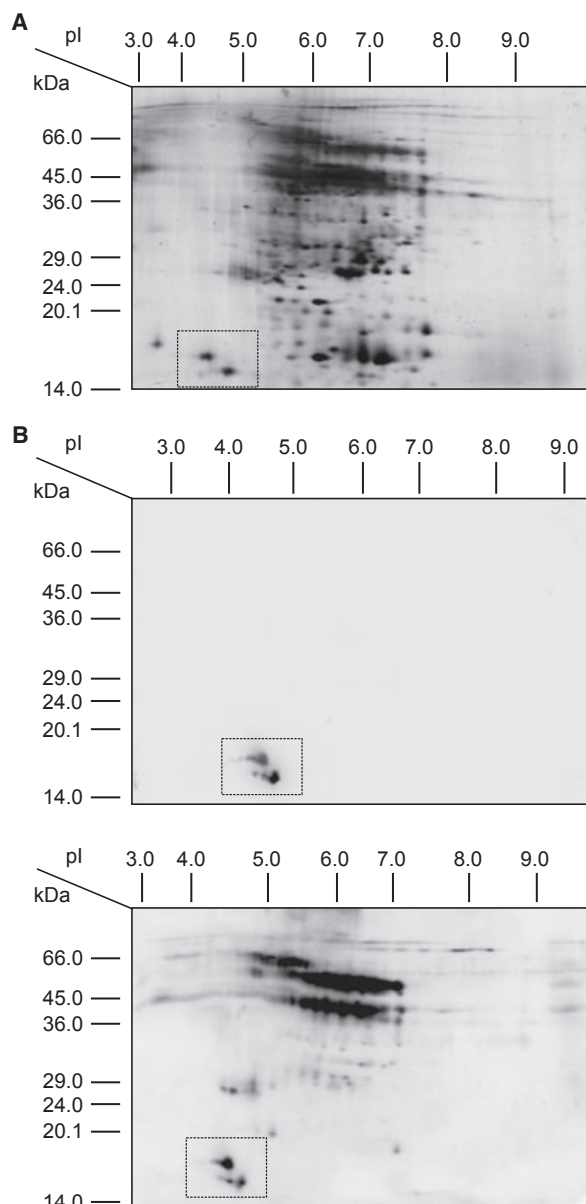


Fig. 1. Identification of profilin by 2D-PAGE analysis of *Salsola kali* pollen extract. (A) Silver staining of the pollen extract. (B) Immunostaining of *S. kali* protein extract after transfer to nitrocellulose membranes using a polyclonal antiserum specific to profilin (upper panel), or a pool of five sera from sensitized patients to Chenopodiaceae pollen (lower panel). Molecular mass markers are indicated.

polyclonal antiserum (Fig. 1B). This protein appears as a group of defined IgG-binding spots between 14.1 and 15.9 kDa and with pI values of 4.4, 4.5 and 4.7. The allergenic character of the protein was confirmed using a pool of sera from patients sensitized to profilin (Fig. 1C). The relative abundance of the isoforms and the IgG-binding profile were very similar (Fig. 1A,B). However, a different IgE-binding pattern was observed, as the two less intense spots of high molecular weight that display lower IgG recognition were better recognized by the IgE of *S. kali*-sensitized patients.

Cloning, recombinant production and purification of the *S. kali* profilin isoforms

cDNA was synthesized from *S. kali* pollen total RNA and used directly in two successive rounds of PCR to

clone *S. kali* profilin. The first round of PCR was performed using a specific primer based on the N-terminus amino acid sequence MSWQA/TYV obtained by Edman degradation of the natural protein. After sequencing the first-round PCR product, we obtained the whole cDNA sequence using specific primers based on the N- and C-terminal ends of *S. kali* profilin. We obtained two isoforms, Sal k 4.02 and Sal k 4.03 (Fig. 2A). Sal k 4.02 showed 26 amino acid changes compared with Sal k 4.03, and 33 amino acid differences compared with Che a 2 (the profilin from *C. album*), 17 of which were non-conservative changes. Eight of the non-conservative changes for Sal k 4.02 (A5, S39, N40, F68, E72, A83, C95 and T100) and Sal k 4.03 (A5, A39, N40, H68, T72, Q83, C95 and G100) were located in the IgE epitopes previously described for the homologous plant profilin Cuc m 2 [25]. We also observed that Sal k 4.03 contains two

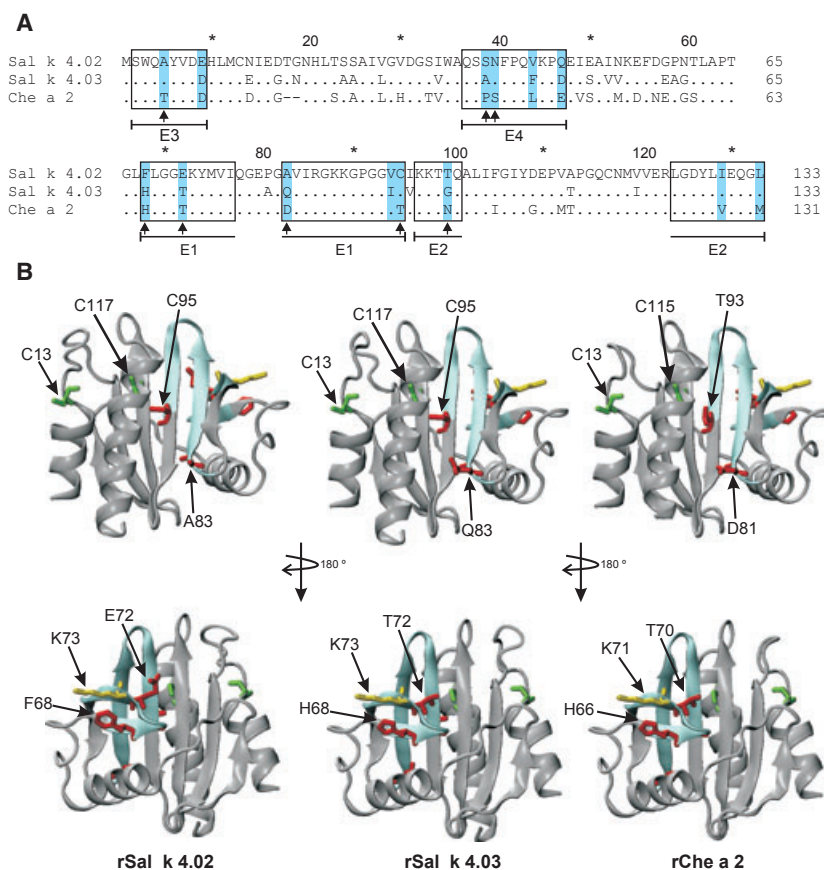


Fig. 2. Amino acid sequence alignment and 3D structure modeling of *Salsola kali* and *Chenopodium album* profilins. (A) Amino acid alignment of Sal k 4.02, Sal k 4.03 and Che a 2. The four epitopes (E1–E4) identified in Cuc m 2 profilin [25] are boxed, and the amino acid differences are highlighted in blue. Arrows indicate non-conserved amino acid changes inside the epitopes; dots indicates the same amino acid as rSal k 4.02; hyphens indicate gaps. (B) 3D modeling of Sal k 4.02, Sal k 4.03 and Che a 2. The E1 epitope is shown in pale blue, the non-conservative amino acid changes are indicated in red, and the conserved K73/71 amino acids are indicated in yellow. Cysteine residues located outside the E1 epitope are shown in green.

non-conservative changes inside the epitope E1 with respect to Che a 2 (H68 and T72). Furthermore, profilin isoforms from *S. kali* possess a third cysteine residue at position 95 compared to Che a 2, which contains threonine at this position (Fig. 2B).

We expressed both isoforms in *E. coli* to determine whether the eight non-conservative changes in Sal k 4.02 at the E1, E2, E3 and E4 epitopes were responsible for decreased IgE-binding capacity. The highest level of recombinant proteins was observed after 4 h of induction with 0.4 mM isopropyl thio- β -D-galactoside (Fig. 3A). Recombinant proteins were observed in the soluble fraction after cell disruption, and were detected using a specific polyclonal antiserum against profilin (Fig. 3B). Supernatant containing profilin was lyophilized, and subjected to size-exclusion chromatography followed by reversed-phase HPLC (Fig. 3C).

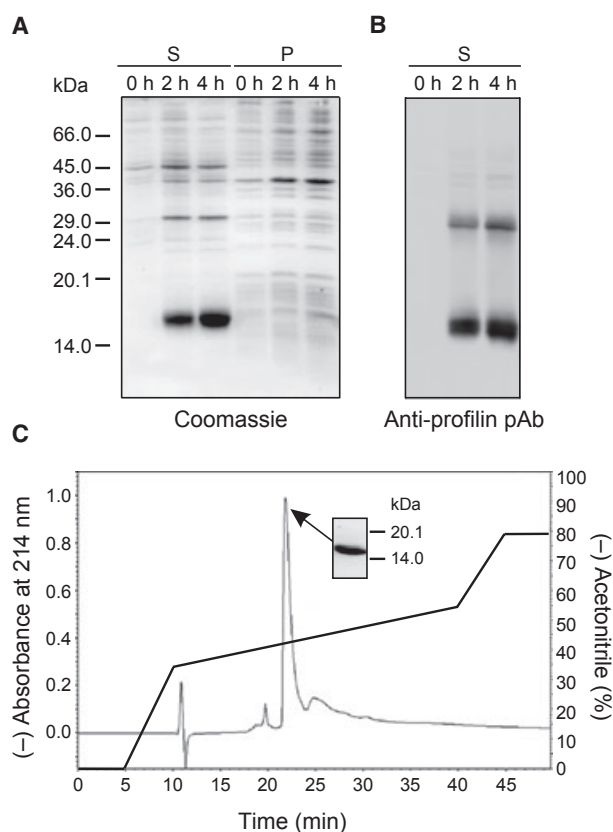


Fig. 3. Recombinant production of *Salsola kali* profilins. (A) SDS/PAGE analysis of the time course of rSal k 4.02 expression. S, soluble fraction; P, pellet. The same expression profile was obtained for rSal k 4.02 and rSal k 4.03. (B) Time-course expression of rSal k 4 in the soluble fraction was also analyzed by immunostaining with profilin-specific polyclonal antiserum after SDS/PAGE. (C) Reversed-phase HPLC purification profile of the recombinant profilins and SDS/PAGE analysis of the fractions collected (inset). Molecular mass markers are indicated.

Molecular characterization of rSal k 4 isoforms

Molecular characterization of rSal k 4.02 and rSal k 4.03 was performed in comparison with rChe a 2. Molecular mass was determined by mass spectrometry. The experimental values for the MS of rSal k 4.02 and rChe a 2 are 138.4 and 123.7 Da lower, respectively, than the theoretically estimated masses derived from the cDNA sequences of the clones (14 340.3 and 14 099.1 Da, respectively) (Fig. 4A), suggesting that these two proteins have lost their initial methionine by translational processing. However, bacterial processing of the N-terminus of rSal k 4.03 had not taken place as the theoretical mass was 14 268.2 Da and a value of 14 213.7 Da was obtained experimentally by MS (Fig. 4A). The amino acid compositions obtained by acid hydrolysis of the three recombinant proteins agree with those derived from the DNA sequences.

Secondary structure analyses were performed by far-UV CD. Similar spectra and percentages of secondary structure elements were obtained for rSal k 4.02, rSal k 4.03 and rChe a 2 (Fig. 4B) as deduced by deconvolution of the spectra (Fig. 4C), and corresponded to the parameters obtained for the 3D structure of the profilin from *Arabidopsis thaliana* [26]. These data indicate that the recombinant proteins rSal k 4.02 and rSal k 4.03 are properly folded.

The electrophoretic mobility of the three profilins was assayed in the presence or absence of 2-mercaptoethanol (Fig. 4D). The mobility of rChe a 2, unlike that of rSal k 4.02 and rSal k 4.03, was significantly affected under reducing conditions. This effect was probably due to the potential formation of a disulfide bond in *S. kali* profilins between cysteines 95 and 117 instead of the disulfide bond observed in Che a 2 between cysteines 13 and 117. Disruption of this disulfide bond in *S. kali* profilins after reduction does not induce a significant conformational change, and therefore the electrophoretic mobility is not affected.

Immunological characterization: rSal k 4.02 possess low IgE-binding ability

An ELISA was used to evaluate the IgE-binding ability of rSal k 4.02 and rSal k 4.03, and to establish the prevalence of these allergens in a population of 165 patients with Chenopodiaceae pollinosis. The prevalence of rChe a 2 and rSal k 4.03 was 29%, and that of rSal k 4.02 was 24%. Moreover, 86% of the sera that showed positive IgE-binding values for the three profilins showed higher reactivity against rSal k 4.03 and rChe a 2 than to rSal k 4.02 (Fig. 5A). The same sera showed significant differences in IgE-binding

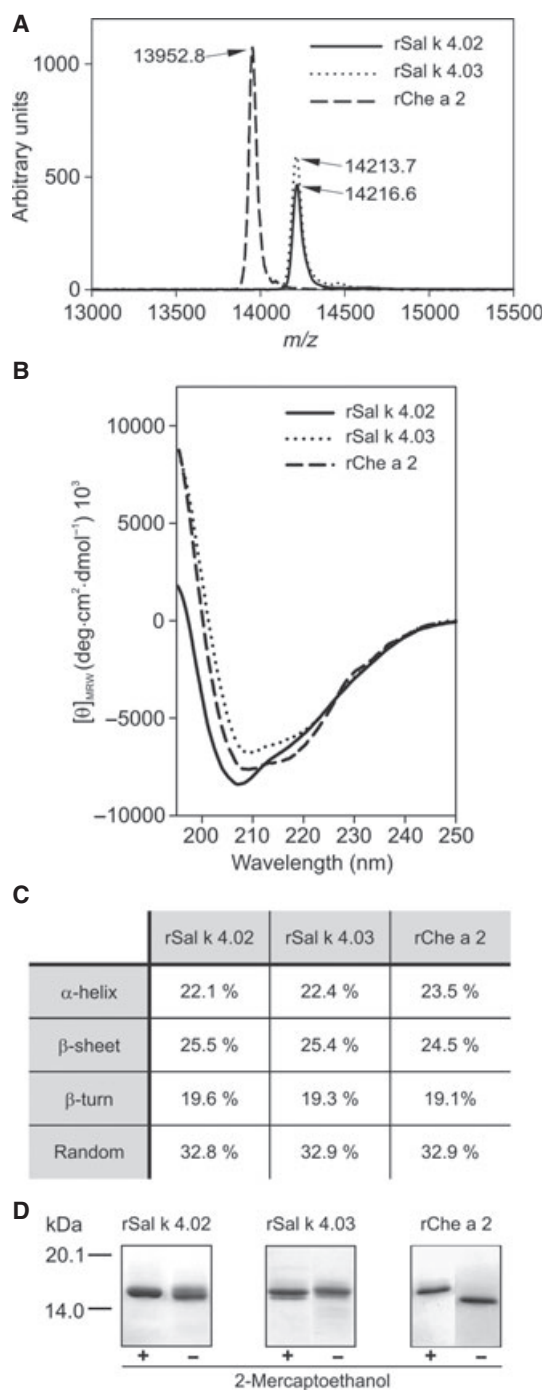


Fig. 4. Molecular characterization of rSal k 4.02 and rSal k 4.03 in comparison with rChe a 2. (A) MS analysis of purified recombinant proteins. m/z , mass/charge ratio. (B) CD spectra in the far-UV range (195–250 nm). $[\theta]_{MRW}$, mean residue weight ellipticity. (C) Deconvolution values determined from CD spectra of each protein. (D) Analysis of the mobility of rChe a 2, rSal k 4.02 and rSal k 4.03 proteins in the absence (–) or presence (+) of 2-mercaptoethanol by SDS/PAGE and Coomassie Blue staining. Molecular mass markers are indicated.

capacity when comparing rSal k 4.02 with rSal k 4.03 and rSal k 4.02 with rChe a 2, the median reduction of rSal k 4.02 IgE-binding capacity being $\sim 35\%$ ($P < 0.05$), but not when comparing rSal k 4.03 with rChe a 2 ($P = 0.72$) (Fig. 5B).

We tested five randomly collected sera by immunoblotting against rChe a 2, rSal k 4.02 and rSal k 4.03 under reducing conditions (Fig. 5C). Similar differences in the IgE recognition pattern were observed for both ELISA and immunoblotting. These data suggest that rSal k 4.02 is not able to bind IgE antibodies as well as rSal k 4.03 and rChe a 2.

Profilin-specific polyclonal antiserum allowed us to evaluate the antigenic character of these allergens in comparison with rChe a 2. In contrast to the decreased IgE-binding observed for rSal k 4.02, inhibition assays performed with anti-profilin polyclonal antiserum against the three allergens showed a similar IgG-binding behavior (Fig. 6A). An inhibition assay performed with a pool of eight human sera showed a sharp decrease in the inhibition capacity of rSal k 4.02 compared with rSal k 4.03 and rChe a 2 (Fig. 6B). The IgE-binding inhibition of rSal k 4.03 by rSal k 4.02 required 35 times as much inhibitor to reach the IC_{50} inhibition. Moreover, to achieve the same inhibition to rChe a 2, nine- and 100-fold increases in the inhibitors rSal k 4.03 and rSal k 4.02 over Che a 2 were required, respectively. rChe a 2 and rSal k 4.03 were able to totally inhibit IgE binding to rSal k 4.02. However, inhibition reached similar values with all profilins used as inhibitors when their concentration was ten times higher than the antigen in the wells and IgE binding was saturated with the inhibitors.

Thus these results indicate that rSal k 4.02 is a natural hypoallergenic profilin isoform in comparison to rSal k 4.03 and rChe a 2, whose IgE-binding behavior was almost equivalent.

Discussion

The production of hypoallergenic derivatives from modified cDNA encoding allergens is an important strategy that is currently used to obtain new molecules with improved qualities for use as diagnostic and/or therapeutic tools in clinical trials. Successful allergen-specific immunotherapy is associated with an increase in blocking IgG antibodies [27]. Many approaches have been used to obtain allergenic variants with decreased IgE-binding capacity that are able to induce an IgG-associated response. Several strategies involve disrupting the 3D structure and thus the conformational B-cell epitopes but maintaining the T-cell epitopes, such as deletion variants [28], fragments and

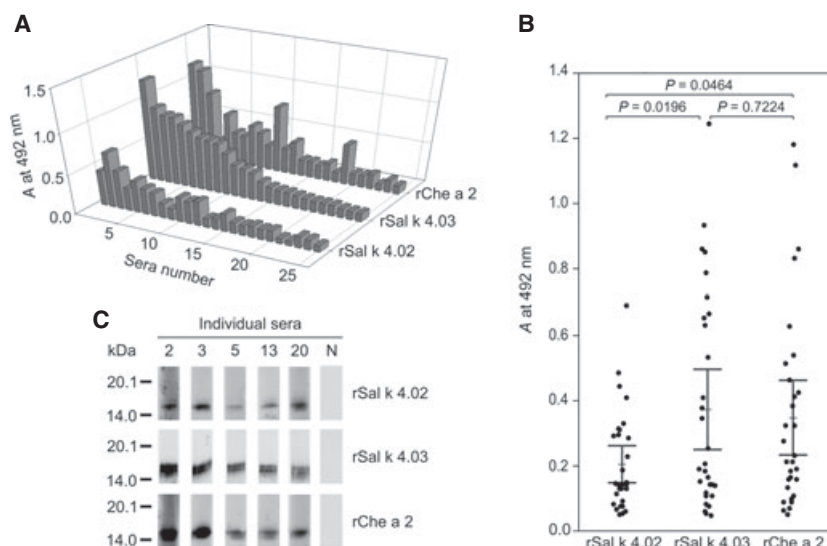


Fig. 5. Analysis of the IgE binding to rSal k 4.02, rSal k 4.03 and rChe a 2. (A) Specific IgE levels for each allergen in absorbance units for 25 representative individual sera in ELISA. (B) Median value and standard deviation of all sera tested in the assay. *P* values for all comparisons were calculated. (C) Analysis of IgE binding to nitrocellulose-blotted samples after SDS/PAGE using sera from five allergic patients and a non-atopic control (lane N). Molecular mass markers are indicated.

allergen-tandem derivatives [29–32], mutants and hybrid molecules [22]. Others are based on producing allergens in which the IgE-binding residues have been changed while preserving the correct folding of the molecule [33–35]. Such strategies have had various levels of success, but in all cases artificial molecules such as chimeras or non-natural isoforms have been used. The potential advantages of natural hypoallergenic molecules over genetically modified ones are that they are more likely to achieve a 3D fold identical to that of the sensitizing allergen. Thus, the availability of recombinant isoforms of an allergen with equivalent antigenic but lower allergenic properties may enable identification of the features that make an allergen a molecule able to stimulate the immune system, in addition to its clinical potential.

The widespread distribution of *S. kali* in arid regions and countries suffering from desertification means that this weed is an important source of allergenic pollen. Recently, an increase in sensitization to this plant has been observed in several regions of Spain, with Sal k 1 being the only major allergen identified and a marker for primary sensitization to this pollen [7].

The growing number of patients who are polysensitized to various pollens has emphasized the importance of pan-allergens. Many reports have focused on these ubiquitous allergens because they are responsible for a high number of polysensitization diagnoses, even though these individuals may be sensitized to a single allergen family [18,19,36]. Profilins are proteins that

have been identified in all analyzed eukaryotic cells and show a highly conserved amino acid sequence, explaining their frequent involvement in IgE polysensitization. Indeed, profilins have been implicated in cross-reactivity between pollens and fruits and in the latex fruit syndrome [18,19,37–39]. The amount of the natural protein in many biological sources such as Chenopodiaceae pollen is quite low. As previously described, 30 µg of pure profilin may be obtained per gram of dried Chenopod pollen [10]. In addition, this low amount of protein is often present in several isoforms with different allergenic features. To solve this yield problem, heterologous expression systems may be used to obtain reasonable amounts of recombinant protein. High-scale production may improve structural and immunological characterization, but limits study to few or just one isoform of the allergen instead of the multiple isoforms that frequently occur in the natural source. The availability of recombinant allergens in diagnosis and treatment of allergic disease encourages the production of modified genetically engineered allergens.

The allergens Sal k 4.02 and Sal k 4.03 described here were produced in *E. coli* and isolated using several chromatographic steps. Purity of the final products was achieved by MS, reversed-phase HPLC and SDS/PAGE. The molecular characterization and analysis of their amino acid sequences showed typical properties of profilins, such as an acidic isoelectric point close to 4.5, a molecular mass of ~14 kDa, and

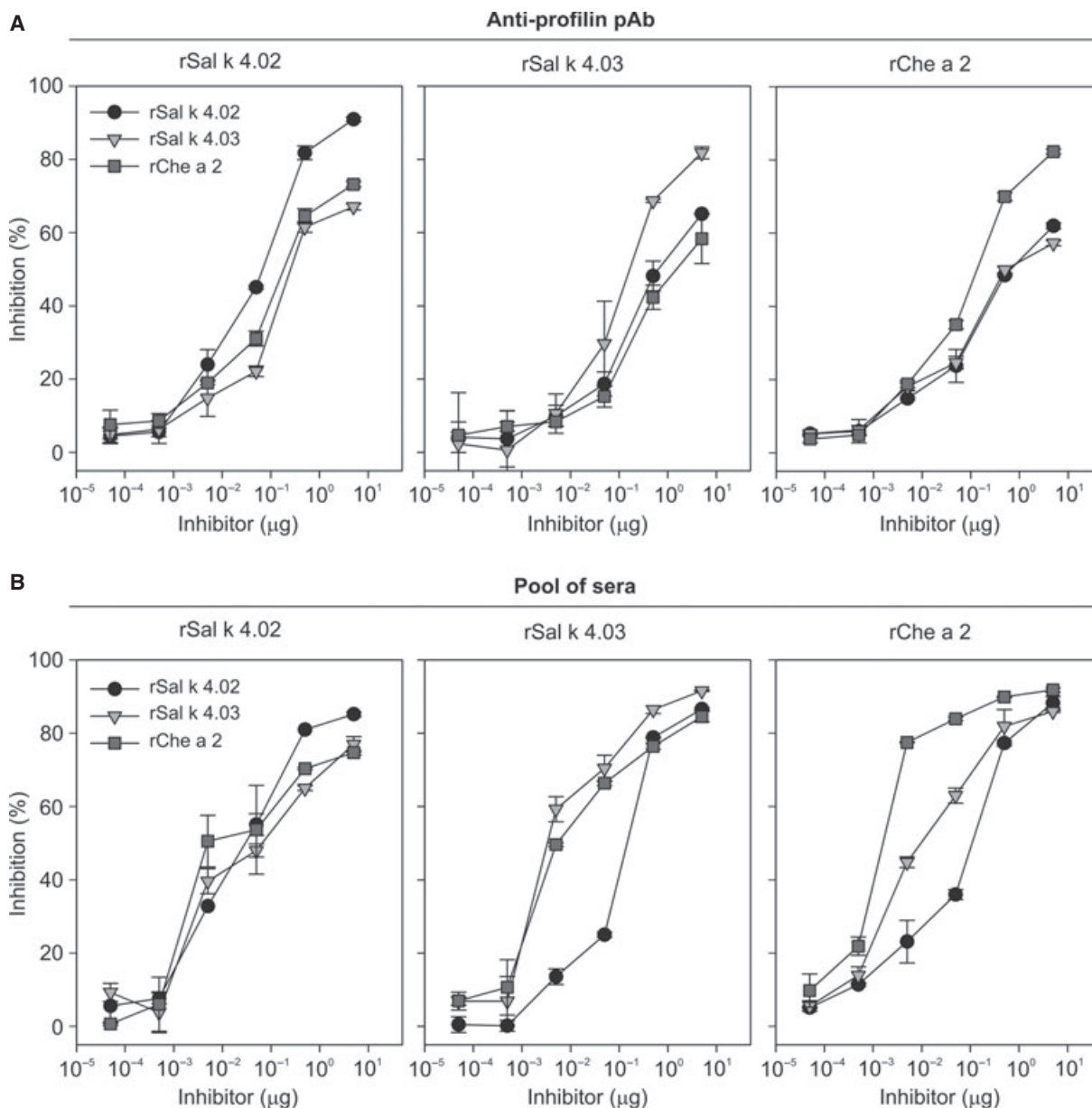


Fig. 6. ELISA inhibition test with rSal k 4.02, rSal k 4.03 and rChe a 2. (A) Polyclonal antibody-binding inhibition to rSal k 4.02, rSal k 4.03 and rChe a 2-coated wells using the same allergens as inhibitors. (B) ELISA inhibition assay of a pool of sera from eight random sensitized patients to rSal k 4.02, rSal k 4.03 and rChe a 2-coated wells, using the same allergens as inhibitors.

a secondary structure content similar to other members of the family. The recombinant profilins obtained from *S. kali* pollen showed a notable degree of amino acid sequence identity with other identified profilins. To compare the immunological features of *S. kali* profilin isoforms, rChe a 2 profilin from the botanically related plant *C. album* was used as reference [8], as rChe a 2 had been previously validated against the natural allergen isolated from *C. album* pollen [10]. After immunological characterization, we determined

that the various isoforms of Sal k 4 show various IgE-binding capabilities, and suggests a hypoallergenic nature for rSal k 4.02 in comparison to rSal k 4.03 and rChe a 2 because it binds significantly lower levels of IgE. Although the possibility cannot be discarded that this response is due to a lower IgE cross-reactivity of Sal k 4.02 to other primary sensitizing profilins in other pollens of the environment, it should be taken into account that *S. kali* pollen is a main source of sensitization for the Zaragoza patient population [40],

and thus *S. kali* profilin is a primary sensitization source. In the province of Jaén, *S. kali* co-exists with olive tree cultivars, the main agriculture crop, and is an important source of allergenic pollen.

The assayed profilins from *S. kali* and *C. album* show a high amino acid sequence identity (73% between rSal k 4.02 and rChe a 2, 75% between rSal k 4.03 and rChe a 2 and 80% between rSal k 4.02 and rSal k 4.03) and similarity (87%, 90% and 87%, respectively). Interestingly, the previously reported profilin from *S. kali*, Sal k 4.01 [41] shows higher identity and similarity to rChe a 2 (78% and 91%, respectively) than rSal k 4.02 or rSal k 4.03 do, probably as a consequence of using degenerate primers derived from comparisons with other plant profilins, and highlighting the polymorphic character of *S. kali* profilin. These data support the different allergenic character of rSal k 4.02, with the immunological behavior of rSal k 4.01 being more similar to rChe a 2 [41].

The three profilins studied here share common IgG and IgE epitopes because almost all the positive patients recognizing rChe a 2 are also positive for *S. kali* profilins, and near complete inhibition of the IgE-binding signal to rSal k 4.02 and rSal k 4.03 is obtained when rChe a 2 is used as inhibitor. However, as deduced from inhibition assays using a pool of sera, 35- and 100-fold increases in rSal k 4.02 were required to inhibit IgE binding to rSal k 4.03 and rChe a 2, respectively. On the other hand, only a ninefold increase in rSal k 4.03, which shares some of the amino acid changes with rChe a 2 (F68→H66 and E72→T70) and contains C95 in the E1 epitope as for rSal k 4.02, was required to inhibit IgE binding to rChe a 2. Due to the high similarity between these profilins (even higher in the epitopic regions), and assuming that the secondary structure of rSal k 4.02 obtained by CD analysis is that expected for a well-folded state in this protein family, the decreased immunological features are associated with several amino acid replacements in the IgE epitope sequences. Thus, the affinity changes produced by a few key amino acid replacements are the main cause of alterations in the immunological response. These alterations are mitigated at very high concentration of inhibitors that lead to complete inhibition.

Thirty-three and 29 amino acid changes were observed between Che a 2 and Sal k 4.02 and between Che a 2 and rSal k 4.03 respectively, 17 of which were non-conservative, of which eight were located within the epitopes (comparing Che a 2 with Sal k 4.02). The E1, E2, E3 and E4 epitopes show four, one, one and two non-conservative changes, respectively (Fig. 2A). However, all the changes present in the E2, E3 and E4 epitopes and the A at position 83 of the E1 epitope in

Sal k 4.02 are present in other allergenic profilins such as those from *Olea europaea*, *Betula pendula* or *Zea mays* [42], and thus are unlikely to be the cause of the reduced allergenicity. However, the E1 epitope binds IgE mainly through electrostatic interactions [43,44] and shows two changes in charge in Sal k 4.02 (F68→H66 and E72→T70) that are adjacent to K73 in Sal k 4 and K71 in Che a 2, which is an amino acid highly conserved in most allergenic profilins. Furthermore, the E1 epitope shows one additional non-conservative amino acid change (C95→T93) that may result in formation of a disulfide bond with C117, resulting in a more compact structure in the surrounding region. These changes may be enough to avoid IgE binding to rSal k 4.02 throughout the E1 epitope, thus resulting in a natural variant with reduced IgE-binding capacity in comparison to rChe a 2. Thus, the results obtained here indicate that some amino acid positions may be critical for IgE binding but not IgG binding.

Although to date there are no immunotherapeutic approaches based on profilin alone and its potential use requires further study, we suggest that, based on the results presented here, the isoform Sal k 4.02 may be a good candidate for inclusion in desensitization protocols using cocktails of recombinant allergens.

Experimental procedures

Protein extracts

Pollen from *S. kali* and *C. album* were obtained from ALK-Abelló (Madrid, Spain). Pollen protein extracts were obtained by saline extraction as described previously [45]. The protein extract concentration was determined as described by Lowry *et al.* [46].

Cloning strategy

cDNA from *S. kali* pollen was synthesized from total RNA using a SMART RACE cDNA amplification kit (BD Biosciences/Clontech, Madrid, Spain) according to the manufacturer's instructions, and used directly as a template for PCR cloning. Amplification of *S. kali* profilin was performed in two successive rounds of PCR. The sense oligonucleotide was designed based on the N-terminus amino acid sequence MSWQA/TYV, obtained by Edman degradation of the natural protein, including the *Nde*I restriction site (underlined): 5'-atacatATGTCNTGGCARRCNTAYGT-3'. A PCR reaction was performed using this oligonucleotide and the universal primer mix contained in the SMART RACE cDNA amplification kit to obtain the *S. kali* profilin cDNA sequence. Then, the antisense oligonucleotide 5'-cgctgaggatccTTAGAGGCCCTGTTCAACGAG-3', containing a *Bam*HI restriction site (underlined),

was designed based on the sequence corresponding to the C-terminus end (LVEQGL) of *S. kali* profilin obtained from the first round of PCR. PCR products were cloned into pCR2.1 plasmid (Invitrogen, Groningen, The Netherlands), and the DNA from several clones was sequenced. The pCR2.1/Sal k 4 constructs were digested with *Nde*I and *Bam*HI restriction enzymes, and cDNA encoding the Sal k 4 isoforms was sub-cloned into the pET 11b (Novagen, Billerica, MA, USA) expression vector digested using the same restriction enzymes. This construct was used to transform *E. coli* BL21(DE3) cells to express Sal k 4.02 and Sal k 4.03 as non-fusion recombinant proteins.

Expression and purification of rSal k 4.02 and rSal k 4.03

Overnight BL21 (DE3) cultures containing the recombinant constructs were grown in LB medium containing 0.1 mg/ml ampicillin to an attenuation at 600 nm of 1.0. Induction of these cultures was performed by the addition of 0.4 mM isopropyl thio- β -D-galactoside, and they were maintained for 4 h at 37 °C. Then the cultures were centrifuged at 6000 *g* for 20 min at 4 °C. The pellet was reconstituted in 20 mM ammonium bicarbonate, pH 8.0, containing 1 mM phenylmethanesulfonyl fluoride. After bacterial lysis by three cycles of freezing and thawing at 42 °C, the material was centrifuged at 12 000 *g* and the soluble fraction was lyophilized. The recombinant protein was isolated as described for rChe a 2 [10], and purified in three chromatographic steps: size-exclusion chromatography using a Sephadex G-50 medium column (39 $\times$ 5.75 cm) equilibrated in 0.2 M ammonium bicarbonate, pH 8.0, size-exclusion chromatography in a Sephadex G-50 superfine column (42 $\times$ 2 cm) equilibrated in the same buffer, and finally reversed-phase HPLC in a Nucleosil C18 column with an 0–80% acetonitrile gradient in 0.1% trifluoroacetic acid.

Sera and antibodies

The Ethical Committee of the Complutense University (Madrid, Spain) approved the protocols used for experimental work with mice and all the methodology related to the use of human sera in the study.

Sera were obtained from patients fulfilling the following criteria: (a) seasonal rhinitis and/or asthma from April to August during at least two consecutive years, (b) positive prick test for *S. kali* and/or *C. album* pollens, and (c) IgE values by ELISA against Che a 2 higher than 0.1 units of absorbance at 492 nm for all sera. Sera were obtained from 165 patients recruited from Zaragoza (Spain), where *S. kali* pollen is a main source of sensitization, and from Jaén (Spain), where *S. kali* pollen is a significant allergenic pollen. Written informed consent was obtained from all patients.

Rabbit polyclonal antiserum against the olive pollen profilin (Ole e 2) was obtained by weekly injections of the

protein (100 μ g) in complete Freund's adjuvant. The use of animals for experimental procedures was supervised by the animal facility of the Fundación Jiménez Díaz (Madrid, Spain) to ensure that the experiments were performed in accordance with legislation and the guidelines of Ethical Committee of the Fundación Jiménez Díaz and Complutense University. Horseradish peroxidase-labeled goat polyclonal Ig against rabbit IgG was obtained from Bio-Rad (Richmond, CA, USA). Mouse monoclonal Ig against human IgE was kindly donated by ALK-Abelló. Horseradish peroxidase-labeled goat polyclonal Ig against mouse IgG was purchased from Pierce Chemical Co (Rockford, IL, USA).

Edman degradation, MS analysis and CD

Edman degradation of the protein was performed using a 477A sequencer (PE Applied Biosystems, Foster City, CA). MS of purified proteins was performed in a Bruker Reflex IV MALDI-TOF apparatus (Bruker-Franzer Analytic, Bremen, Germany). The CD spectrum was recorded in the far-UV range on a JASCO J-715 spectropolarimeter (Japan Spectroscopic Co., Tokyo, Japan) at 200 μ g·mL⁻¹ protein concentration in 50 mM sodium phosphate, pH 7.5, at room temperature. CDNN CD spectra deconvolution software (Applied Photophysics, Leatherhead, UK) was used to deconvolute the CD spectra recorded.

Analytical procedures

Amino acid analysis of pure recombinant proteins was performed using a Biochrom 30 amino acid analyzer instrument (Biochrom, Cambridge, UK) after hydrolysis with 6 N HCl. SDS/PAGE was performed in 17% polyacrylamide gels. Proteins were stained with Coomassie Blue R-250 (Sigma-Aldrich, St Louis, MO). Molecular mass determinations were performed using protein markers MW-SDS-70L (Sigma-Aldrich). 2D electrophoresis was performed in a PROTEAN IEF cell (Bio-Rad) using 7 cm long, pH 3–10, linear ReadyStrip IPG strips (Bio-Rad) under reducing conditions in the presence of tributylphosphine, dithiothreitol and iodoacetamide, followed by 17% SDS/PAGE. Proteins were visualized after silver staining.

The concentration of recombinant profilins was calculated by measuring the absorbance at 280 nm in a DU-7 spectrometer (Beckman, Barcelona, Spain) using the same extinction coefficient ($\epsilon^{0.1\%}$) of 1.1 for the three proteins.

Immunological characterization

Proteins were transferred to nitrocellulose membranes (Amersham Biosciences, Barcelona, Spain) after SDS/PAGE under reducing (with 5% 2-mercaptoethanol) and non-reducing conditions or after 2D electrophoresis under reducing conditions. Membranes were incubated with individual

human sera (diluted 1 : 10), mouse monoclonal Ig against human IgE (diluted 1 : 5000) and horseradish peroxidase-labeled goat Ig against mouse IgG (diluted 1 : 2500), or alternatively rabbit polyclonal antiserum raised against Ole e 2 (diluted 1 : 10 000) followed by horseradish peroxidase-labeled goat Ig against rabbit IgG (diluted 1 : 3000). For immunoblotting inhibition assays, a pool of sera ($n = 5$) (diluted 1:10) was pre-incubated with NaCl/P_i and 1 or 5 µg of rSal k 4.02, rSal k 4.03 or rChe a 2 as inhibitors. Chemiluminescent signal was detected using ECL western blotting reagent (Amersham Biosciences).

Indirect ELISA was performed in 96-well plates coated with 0.1 µg purified recombinant protein or 20 µg pollen extract. Assays were detected using individual human serum, a pool of human sera ($n = 5$) (diluted 1 : 10) or anti-Ole e 2 polyclonal antiserum (diluted 1 : 10 000), previously adsorbed to various concentrations of inhibitors ranging from 1 ng to 10 µg. Binding of human IgE was detected by mouse monoclonal Ig against human IgE (diluted 1 : 5000) followed by horseradish peroxidase-labeled goat Ig against mouse IgG (diluted 1 : 2500), and binding of polyclonal antiserum against Ole e 2 was detected by horseradish peroxidase-labeled goat Ig against rabbit IgG (diluted 1 : 3000). The signal was measured at 492 nm in an iMark microplate absorbance reader (Bio-Rad). In ELISA assays, absorbance values above 0.1 were taken as positive, and the percentage inhibition was calculated according to the formula: inhibition (%) = $[1 - (\text{absorbance at 492 nm with inhibitor} / \text{absorbance at 492 nm without inhibitor})] \times 100$.

Statistical analysis, modeling and alignment

For analysis of ELISA datasets, we used JMP 10 (SAS Business Analytics) to calculate the mean, standard deviation and P values. A two-tailed Student's t -test was performed assuming unequal variances to assess whether the groups were statistically different from each other. All P values were derived from a two-tailed statistical test. P values < 0.05 were considered statistically significant. 3D structure modeling of the allergens was performed using SWISS-MODEL, the fully automated protein structure homology-modeling server of the Swiss Institute of Bioinformatics (<http://swissmodel.expasy.org/>) [47]. The template used for modeling Sal k 4.02, Sal k 4.03 and Che a 2 was the profilin from *Arabidopsis thaliana* (Protein Data Bank ID 3NUL) using to the comparative automated mode. The model obtained was represented using VMD [48]. Alignment of sequences was performed using GeneDoc (<http://www.psc.edu/biomed/genedoc>).

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References

- 1 Eder W, Ege MJ & von Mutius E (2006) The asthma epidemic. *N Engl J Med* **355**, 2226–2235.
- 2 Asher MI, Montefort S, Björkstén B, Lai CK, Strachan DP, Weiland SK & Williams H (2006) Worldwide time trends in the prevalence of symptoms of asthma, allergic rhinoconjunctivitis, and eczema in childhood: ISAAC Phases One and Three repeat multicountry cross-sectional surveys. *Lancet* **368**, 733–743.
- 3 Sibbald B (1993) Epidemiology of allergic rhinitis. *Monogr Allergy* **31**, 61–79.
- 4 Dahl R, Andersen PS, Chivato T, Valovirta E & de Monchy J (2004) National prevalence of respiratory allergic disorders. *Respir Med* **98**, 398–403.
- 5 Barber D, de la Torre F, Feo F, Florido F, Guardia P, Moreno C, Quirarte J, Lombardero M, Villalba M, Salcedo G *et al.* (2008) Understanding patient sensitization profiles in complex pollen areas: a molecular epidemiological study. *Allergy* **63**, 1550–1558.
- 6 Colás C, Monzón S, Venturini M & Lezaun A (2006) Double-blind, placebo-controlled study with a modified therapeutic vaccine of Salsola kali (Russian thistle) administered through use of a cluster schedule. *J Allergy Clin Immunol* **117**, 810–816.
- 7 Barderas R, García-Selles J, Salamanca G, Colás C, Barber D, Rodríguez R & Villalba M (2007) A pectin methylesterase as an allergenic marker for the sensitization to Russian thistle (Salsola kali) pollen. *Clin Exp Allergy* **37**, 1111–1119.
- 8 Barderas R, Villalba M, Pascual CY, Batanero E & Rodríguez R (2004) Profilin (Che a 2) and polcalcine (Che a 3) are relevant allergens of Chenopodium album pollen: isolation, amino acid sequences, and immunologic properties. *J Allergy Clin Immunol* **113**, 1192–1198.
- 9 Jockusch BM, Murk K & Rothkegel M (2007) The profile of profilins. *Rev Physiol Biochem Pharmacol* **159**, 131–149.
- 10 Barderas R, Villalba M & Rodríguez R (2004) Recombinant expression, purification and cross-reactivity of chenopod profilin: rChe a 2 as a good marker for profilin sensitization. *Biol Chem* **385**, 731–737.
- 11 Postigo I, Guisantes JA, Negro JM, Rodríguez-Pacheco R, David-García D & Martínez J (2009) Identification of 2 new allergens of Phoenix dactylifera using an immunoproteomics approach. *J Invest Allergol Clin Immunol* **19**, 504–507.

- 12 Valenta R, Duchêne M, Pettenburger K, Sillaber C, Valent P, Bettelheim P, Breitenbach M, Rumpold H, Kraft D & Scheiner O (1991) Identification of profilin as a novel pollen allergen; IgE autoreactivity in sensitized individuals. *Science* **253**, 557–560.
- 13 Valenta R, Ball T, Vrtala S, Duchêne M, Kraft D & Scheiner O (1994) cDNA cloning and expression of timothy grass (*Phleum pratense*) pollen profilin in *Escherichia coli*: comparison with birch pollen profilin. *Biochem Biophys Res Commun* **199**, 106–118.
- 14 López-Torrejón G, Crespo JF, Sánchez-Monge R, Sánchez-Jiménez M, Alvarez J, Rodríguez J & Salcedo G (2005) Allergenic reactivity of the melon profilin Cuc m 2 and its identification as major allergen. *Clin Exp Allergy* **35**, 1065–1072.
- 15 Cases B, Pastor-Vargas C, Dones FG, Perez-Gordo M, Maroto AS, de las Heras M, Vivanco F & Cuesta-Herranz J (2010) Watermelon profilin: characterization of a major allergen as a model for plant-derived food profilins. *Int Arch Allergy Immunol* **153**, 215–222.
- 16 Cabanos C, Tandang-Silvas MR, Odijk V, Brostedt P, Tanaka A, Utsumi S & Maruyama N (2010) Expression, purification, cross-reactivity and homology modeling of peanut profilin. *Protein Expr Purif* **73**, 36–45.
- 17 Vallier P, Balland S, Harf R, Valenta R & Deviller P (1995) Identification of profilin as an IgE-binding component in latex from *Hevea brasiliensis*: clinical implications. *Clin Exp Allergy* **25**, 332–339.
- 18 Weber RW (2003) Patterns of pollen cross-allergenicity. *J Allergy Clin Immunol* **112**, 229–239. quiz 240.
- 19 Santos A & Van Ree R (2011) Profilins: mimickers of allergy or relevant allergens? *Int Arch Allergy Immunol* **155**, 191–204.
- 20 Sirvent S, Tordesillas L, Villalba M, Díaz-Perales A, Cuesta-Herranz J, Salcedo G & Rodríguez R (2011) Pollen and plant food profilin allergens show equivalent IgE reactivity. *Ann Allergy Asthma Immunol* **106**, 429–435.
- 21 Ferreira F, Wallner M, Breiteneder H, Hartl A, Thalhamer J & Ebner C (2002) Genetic engineering of allergens: future therapeutic products. *Int Arch Allergy Immunol* **128**, 171–178.
- 22 Marazuela EG, Rodríguez R, Barber D, Villalba M & Batanero E (2007) Hypoallergenic mutants of Ole e 1, the major olive pollen allergen, as candidates for allergy vaccines. *Clin Exp Allergy* **37**, 251–260.
- 23 Saarne T, Kaiser L, Grönlund H, Rasool O, Gafvelin G & van Hage-Hamsten M (2005) Rational design of hypoallergens applied to the major cat allergen Fel d 1. *Clin Exp Allergy* **35**, 657–663.
- 24 Reisinger J, Horak F, Pauli G, van Hage M, Cromwell O, König F, Valenta R & Niederberger V (2005) Allergen-specific nasal IgG antibodies induced by vaccination with genetically modified allergens are associated with reduced nasal allergen sensitivity. *J Allergy Clin Immunol* **116**, 347–354.
- 25 Tordesillas L, Pacios LF, Palacín A, Cuesta-Herranz J, Madero M & Díaz-Perales A (2010) Characterization of IgE epitopes of Cuc m 2, the major melon allergen, and their role in cross-reactivity with pollen profilins. *Clin Exp Allergy* **40**, 174–181.
- 26 Thorn KS, Christensen HE, Shigeta R, Huddler D, Shalaby L, Lindberg U, Chua NH & Schutt CE (1997) The crystal structure of a major allergen from plants. *Structure* **5**, 19–32.
- 27 Akdis M & Akdis CA (2009) Therapeutic manipulation of immune tolerance in allergic disease. *Nat Rev Drug Discov* **8**, 645–660.
- 28 García-Ortega L, Lacadena J, Villalba M, Rodríguez R, Crespo JF, Rodríguez J, Pascual C, Olmo N, Onaderra M, del Pozo AM *et al.* (2005) Production and characterization of a noncytotoxic deletion variant of the *Aspergillus fumigatus* allergen Asp f1 displaying reduced IgE binding. *FEBS J* **272**, 2536–2544.
- 29 van Hage-Hamsten M, Kronqvist M, Zetterström O, Johansson E, Niederberger V, Vrtala S, Grönlund H, Grönneberg R & Valenta R (1999) Skin test evaluation of genetically engineered hypoallergenic derivatives of the major birch pollen allergen, Bet v 1: results obtained with a mix of two recombinant Bet v 1 fragments and recombinant Bet v 1 trimer in a Swedish population before the birch pollen season. *J Allergy Clin Immunol* **104**, 969–977.
- 30 Mahler V, Vrtala S, Kuss O, Diepgen TL, Suck R, Cromwell O, Fiebig H, Hartl A, Thalhamer J, Schuler G *et al.* (2004) Vaccines for birch pollen allergy based on genetically engineered hypoallergenic derivatives of the major birch pollen allergen, Bet v 1. *Clin Exp Allergy* **34**, 115–122.
- 31 Linhart B, Mothes-Luksch N, Vrtala S, Kneidinger M, Valent P & Valenta R (2008) A hypoallergenic hybrid molecule with increased immunogenicity consisting of derivatives of the major grass pollen allergens, Phl p 2 and Phl p 6. *Biol Chem* **389**, 925–933.
- 32 Campana R, Vrtala S, Maderegger B, Jertschin P, Stegellner G, Swoboda I, Focke-Tejkl M, Blatt K, Gieras A, Zafred D *et al.* (2010) Hypoallergenic derivatives of the major birch pollen allergen Bet v 1 obtained by rational sequence reassembly. *J Allergy Clin Immunol* **126**, 1024–1031. 1031 e1021–1028.
- 33 Walgraffe D, Mattéotti C, El Bakkoury M, Garcia L, Marchand C, Bullens D, Vandenbranden M & Jacquet A (2009) A hypoallergenic variant of Der p 1 as a candidate for mite allergy vaccines. *J Allergy Clin Immunol* **123**, 1150–1156.
- 34 Rouvinen J, Jänis J, Laukkanen ML, Jylhä S, Niemi M, Päivinen T, Mäkinen-Kiljunen S, Haahtela T, Söderlund H & Takkinen K (2010) Transient dimers of allergens. *PLoS ONE* **5**, e9037.

- 35 Ball T, Linhart B, Sonneck K, Blatt K, Herrmann H, Valent P, Stoecklinger A, Lupinek C, Thalhamer J, Fedorov AA *et al.* (2009) Reducing allergenicity by altering allergen fold: a mosaic protein of Phl p 1 for allergy vaccination. *Allergy* **64**, 569–580.
- 36 Ledesma A, Barderas R, Westritschnig K, Quiralte J, Pascual CY, Valenta R, Villalba M & Rodríguez R (2006) A comparative analysis of the cross-reactivity in the polcalcin family including Syr v 3, a new member from lilac pollen. *Allergy* **61**, 477–484.
- 37 Valenta R, Duchene M, Ebner C, Valent P, Sillaber C, Deviller P, Ferreira F, Tejkl M, Edelman H, Kraft D *et al.* (1992) Profilins constitute a novel family of functional plant pan-allergens. *J Exp Med* **175**, 377–385.
- 38 Daschner A, Crespo JF & Pascual CY (1998) Specific IgE to recombinant vegetal panallergen (rBet v 2) and fruit allergy in pollinic patients. *Allergy* **53**, 614–618.
- 39 Wagner S & Breiteneder H (2002) The latex-fruit syndrome. *Biochem Soc Trans* **30**, 935–940.
- 40 Ferrer L, Carnés J, Rojas-Hijazo B, Lopez-Matas MA, Sobrevía MT & Colás C (2012) Assessing degree of flowering implicates multiple Chenopodiaceae/ Amaranthaceae species in allergy. *Int Arch Allergy Immunol* **158**, 54–62.
- 41 Assarehzadegan MA, Amini A, Sankian M, Tehrani M, Jabbari F & Varasteh A (2010) Sal k 4, a new allergen of *Salsola kali*, is profilin: a predictive value of conserved conformational regions in cross-reactivity with other plant-derived profilins. *Biosci Biotechnol Biochem* **74**, 1441–1446.
- 42 Jimenez-Lopez JC, Morales S, Castro AJ, Volkmann D, Rodríguez-García MI & Alché JdeD (2012) Characterization of profilin polymorphism in pollen with a focus on multifunctionality. *PLoS One* **7**, e30878.
- 43 López-Torrejón G, Díaz-Perales A, Rodríguez J, Sánchez-Monge R, Crespo JF, Salcedo G & Pacios LF (2007) An experimental and modeling-based approach to locate IgE epitopes of plant profilin allergens. *J Allergy Clin Immunol* **119**, 1481–1488.
- 44 Sinha N, Mohan S, Lipschultz CA & Smith-Gill SJ (2002) Differences in electrostatic properties at antibody-antigen binding sites: implications for specificity and cross-reactivity. *Biophys J* **83**, 2946–2968.
- 45 Barderas R, Villalba M, Lombardero M & Rodríguez R (2002) Identification and characterization of Che a 1 allergen from *Chenopodium album* pollen. *Int Arch Allergy Immunol* **127**, 47–54.
- 46 Lowry OH, Rosebrough NJ, Farr AL & Randall RJ (1951) Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**, 265–275.
- 47 Arnold K, Bordoli L, Kopp J & Schwede T (2006) The SWISS-MODEL workspace: a web-based environment for protein structure homology modelling. *Bioinformatics* **22**, 195–201.
- 48 Humphrey W, Dalke A & Schulten K (1996) VMD: visual molecular dynamics. *J Mol Graph* **14**, 33–38, 27–38.