

Title: Two non-specific lipid transfer proteins (nsLTP) from tomato seeds are associated to severe symptoms of tomato-allergic patients

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Abbreviations:

BCA Bicinchoninic acid method

βME 2- mercaptoethanol

HRP Horseradish peroxidase

LTP Lipid transfer protein

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1	nsLTP	Non-specific lipid transfer protein
2	OAS	Oral allergy syndrome
3	OPD	O-phenylenediamine dihydrochloride
4	SPT	Skin prick test

Keywords

Tomato allergy, tomato seeds, nsLTP class 1 Sola l 7, nsLTP class 2 Sola l 6, cross-reactivity.

Abstract

Scope: Tomato has become one of the most consumed vegetables worldwide, but its intake is often accompanied by an increasing risk of inducing allergic symptoms. The aims of the work was the identification of new seed-specific allergens associated to severe symptoms in patients allergic to this edible vegetable.

Methods and Results: We used 22 sera from well-defined tomato-allergic patients. Tomato seed proteins were purified and analysed for biochemical and immunological characterization. A mixture of two associated IgE-binding nsLTPs was purified from tomato seeds. Both allergens, Sola l 7 and Sola l 6, displayed primary structure differences with respect to their counterpart, Sola l 3, from tomato pulp/peel. They retained the ability to bind IgE from 71.4% of patients with severe symptoms. The purified proteins induced positive Basophil Activation Test (BAT) and Skin Prick Test (SPT), and displayed cross-reactivity with homologous allergens from peanut and sunflower seeds, among others.

Conclusions: We herein described two novel allergens from tomato seeds that belong to the nsLTP family classes 1 and 2, respectively. This is the first work associating IgE reactivity to these proteins with severe symptoms of certain tomato-allergic patients. Therefore, they are optimal candidates for clarifying the diagnosis of the tomato allergy.

Sola l 7 and Sola l 6 are two allergenic nsLTPs, class 1 and 2 respectively, which have been purified from tomato seeds. Both allergens displayed primary structure differences with respect to Sola l 3, their LTP counterpart from tomato peel. Their low concentration in the whole extract and their association to severe symptoms in patients allergic to this edible vegetable, makes them optimal candidates for clarifying the diagnosis of the tomato allergy.

Introduction

Food allergy is an important health problem that is gradually growing worldwide. The most prevalent food allergens are those of vegetal origin, being affected approximately 2-4% of the European adult population [1]. Within this idea, in recent publications, tomato (*Solanum lycopersicum*) has been described as one of the most prevalent plant derived-food sensitizer [2, 3] with an allergenic frequency that ranges between 1.5-20.0% in different populations of patients with specific IgE [4, 5]. Particularly, 6.5% of this allergic population is sensitized to tomato fruit in the Spanish Mediterranean region although 80% of these patients still tolerated its consumption [6].

Tomato is a widely produced crop plant and one of the most consumed vegetables worldwide. It belongs to the *Solanaceae*, or nightshade plant family, which has been frequently reported to be allergenic [7]. Although specific allergenic proteins, have been already identified in tomato fruit, such as Sola l 1 (profilin), Sola l 2 (β -fructofuranosidase), Sola l 3 (nsLTP), Sola l 4 (Bet v 1-like), Sola l 5 (ciclophilin) and Sola l 6 (nsLTP) [8,9,10, 11] (www.allergen.org), genuine tomato allergy is infrequent. In most cases, sensitization, positive skin or *in vitro* tests occur in the absence of clinical symptoms and allergy manifestations to this food. These individuals are in fact primarily sensitized to other airborne or food allergen sources which show cross-reactivity [12].

Several cases of food allergies without a positive *in vitro* diagnosis test have been reported. For these patients, different allergens have been specifically identified in the seeds of certain

fruits, kiwi [13], for example. Those studies have allowed identifying a 2S albumin and an 11S globulin as IgE-reactive kiwi seed allergens, showing indeed a significant similarity to other previously reported food allergens. Their presence, exclusively in seeds, is low. Therefore, when the full extract is used, the dilution of these allergens in the protein mixture hampers a correct diagnosis.

In the context of tomato allergy, and taking into account this background, several key facts make plausible the existence of overlooked specific allergens in tomato seeds:

- i) Different seeds from other species are well-recognized as potent inducers of food allergy, as previously shown with the kiwi example [13].
- ii) It is well known that seeds can contain high amounts of very stable allergenic proteins such as pathogenesis-related proteins, TLPs, or LTPs [14], as well as storage proteins like 2S albumins, 7S vicilins, or 11S globulins. All them are potentially capable of sensitizing at the gastrointestinal level [15] and can be involved in the cross-reactivity observed with peanut, tree nuts, and other foods [16].
- iii) From the viewpoint of food handling and manufacturing, seeds are not usually removed from the tomato, and therefore, they are usually ingested together with the pulp.

In summary, searching for the presence of new allergens in tomato seeds appeared as an interesting approach to further improve the diagnosis and, maybe, the treatment of tomato allergy.

Members of the nsLTP plant family are widely cross-reacting plant pan-allergens of 9-10 kDa molecular mass widespread in the plant kingdom. They are small basic cysteine-rich lipid-binding polypeptides with a pI value of around 9 and a primary structure consisting of 91-95 amino acids. Indeed, in good agreement with the features of most food allergens, they usually are very stable proteins [17]. Their function, however, is not clear yet, but they have been implicated in binding of lipids through a compact domain consisting of four α -helices separated by short β -turns and an unstructured C-terminal coil of variable size [18]. Thus, their function consists essentially in facilitating fatty acids transfer between different membrane systems. They are indeed a family of highly resistant proteins to protease digestion.

According to their different molecular masses, the nsLTP family has been divided in two subfamilies: Class 1, containing molecules of around 9 kDa, and class 2, corresponding to

those ones of about 7 kDa. These proteins have been detected in many vegetable sources and are one of the most frequent causes of food-induced anaphylaxis [19]. It has been reported that nsLTPs are predominantly present in the peel of fruits but members from both classes have been also identified in tomato seeds by means of electrospray MS and MALDI imaging [20].

The aim of this study was to identify and characterize two new relevant allergens corresponding to specific nsLTPs from tomato seeds. This study was originated by the upsurge of several patients that reported clinical symptoms when they consumed this fruit with seeds but tolerated tomato when the seeds were removed. In this study, 22 patients with a clinical history of tomato allergy reporting immediate IgE-mediated symptoms after ingestion of this vegetable were included. All these patients presented different symptoms that ranged from the most severe, as anaphylaxis, to mild reactions, such as OAS. The results obtained indicate the existence of a relationship between the sensitization to these nsLTPs and the appearance of severe symptoms in tomato allergic patients. These conclusions might help to improve the current diagnosis of tomato allergy.

Materials & Methods

Patients selection

Tomato allergic patients were recruited from the Allergy Services of Regional University Hospital of Málaga and the Hospital Infanta Leonor of Madrid. Inclusion criteria were a well-defined clinical history of tomato allergy and positive skin prick test to tomato. Several patients referred relief of symptoms when the seeds were removed from tomato. However, most patients included in the study, avoided its consumption after the first reactions. The patients were classified according to their clinical symptoms after tomato intake: mild reactions, including oral allergy syndrome (OAS), moderate reactions, such as urticaria and severe reactions, including anaphylaxis [21]. As negative controls, we included a group of age- and sex-matched individuals with tolerance to tomato and without food allergic episodes.

The study was conducted according to the principles of the Declaration of Helsinki and approved by the Ethic Committee of both hospitals. All participants were informed and

signed the corresponding informed consents before being included in the study. The Ethical Committee of the Complutense University (Madrid, Spain) and Ethical and Research committee of Regional University Hospital of Malaga (#02/2012) approved the protocols used for this experimental work and all the methodology related to the use of human sera in this study.

Skin prick test

SPT were carried out by using tomato extract, tomato, nuts (hazelnut, walnut, peanut, sunflower seed and almond), rosaceae (peach, plum, apple and apricot), fruit-latex (banana, kiwifruit, avocado, fig and chestnut) and Olea pollen, from (ALK-Abello, Madrid, Spain) and natural purified proteins: the tomato seed nsLTPs described in this study (Sola l 6 and Sola l 7), Pru p 3 and Ara h 9 (kindly donated by Dr A Díaz-Perales), and Hel a 3 at 5 and 50 µg/mL. Moreover, home-made pulp and seed tomato extracts were also used at 5 and 50 µg/mL of total protein concentration. Histamine dihydrochloride (10 mg/mL) and physiologic saline solution were used as positive and negative controls respectively. SPT responses were read after 15 min and a wheal size of 3 mm² greater than the negative control was considered positive.

Specific IgE determination

sIgE to tomato (f25) were determined by ImmunoCAP-FEIA in serum samples, according to manufacturer instructions (Thermo Fisher Scientific, Uppsala, Sweden).

Preparation of tomato proteins extracts

Tomato seeds, pulp, peel and placenta were manually separated and then an extract with each tomato tissue was prepared. Peel, pulp and placenta extracts have been obtained with the same protocol as that of tomato seeds. Placenta is defined as the whitish tissue that surrounds tomato seeds, and it was isolated separately from tomato pulp and peel for these experiments. Pulp is the redish meat once discarded peel, seeds and placenta. With this purpose the different parts were separately homogenized in PBS pH 7.4 and left stirring in an ice bath for 4 h. Next, they were centrifuged at 20,000 xg during 30 min at 4 °C. The supernatants were

collected and dialyzed against water for 48 h at °C, using 3.5 kDa pore membranes. Finally, the extracts were lyophilized, dissolved in a convenient volume of 20 mM ammonium bicarbonate and stored at -20 °C until quantification and use.

Analytical procedures

SDS-PAGE was performed in 0.1% SDS-17% polyacrylamide gels under reducing conditions with 2-mercaptoethanol (β ME). Proteins were visualized by means of Coomassie blue staining (CBS) or alternatively transferred to nitrocellulose membranes (Amersham, Hybond, Germany). Protein concentrations were determined using the bicinchoninic acid method (BCA) (Pierce Chemical, Rockford, Ill) [22].

IgE immunoblotting and Dot Blot analyses

Immunodetection of 25 μ g of tomato seed extract and 1 μ g of purified allergenic proteins, applied directly or after transference from SDS-PAGE on nitrocellulose membranes, was performed using sera from patients allergic to tomato (diluted 1:5), as previously described [23]. Binding of human IgE was detected by using mouse anti-human IgE antibodies (diluted 1:5000); kindly donated by Alk-Abello, followed by horseradish peroxidase (HRP)-labeled rabbit anti-mouse IgG (diluted 1:5000) (DAKO, Denmark). The signal was developed with the chemiluminiscent ECL-Western blotting reagent (Amersham). Volummograms of the reactive bands were analysed by duplicate from two independent experiments by scanning densitometry using UVI Band V97 (UVIttec. St. John's Innovation Center). Dot Blot experiments were performed using samples diluted (0.02 μ g/ μ L) in 20 mM ammonium bicarbonate, pH 8, and applied onto the nitrocellulose membrane (100 μ L) using a Minifold dot-blot system, followed by the immunodetection procedure described for Western blot. For immunoblotting inhibition assays, 1 μ g of purified protein were assayed with a pool of sera (diluted 1:5) from tomato allergic patients with anaphylaxis previously incubated with 300 μ g (2 μ g/ μ L) of extracts from tomato tissues (seeds, placenta, pulp, and peel) and peanut, sunflower seeds, kiwi seeds, mustard seeds, peach, and apple extracts. These extracts were obtained with the protocol described [13]. The rest of steps were as above. The signal was detected using ECL Western blotting reagent (Amersham Biosciences, Barcelona, Spain).

Protein Fractionation by HPLC

The allergenic protein mixture was fractionated using an Ultrasphere reverse-phase C-18 column and a 0.1% TFA/ACN gradient (0-60%) with a flow rate of 0.5 mL/min. Protein elution was monitored by UV absorbance readings at 214 and 280 nm.

Nano-LC-MS/MS Analysis

Protein bands from gels obtained after SDS-PAGE fractionation in reducing conditions and corresponding to those detected by IgE in immunoblotting experiments were extracted and analyzed. The EXQuest Spot Cutter (BIO-RAD) system was used for imaging the gels and pick the selected bands which were then subjected to manual tryptic digestion (12 µg/mL) for 8 h at 37 °C. Gel pieces were washed with 50mM ammonium bicarbonate and lately with ACN. To determine the molecular mass of the peptides resulting from the tryptic digestion, a peptide fingerprint was performed. *De novo* sequencing of some peptides was also possible due to fragmentation processes. This analysis was performed in a spectrometer model 4700 Proteomics Analyzer mass TOF/TOF MALDI, an Autoflex III (Bruker-Daltonics). Calibrations were performed with fragment ion spectra obtained for the proton adducts of a peptide mixture covering the m/z 800-3200 region. MALDI-MS and MS/MS data were combined through the BioTools 3.0 program (Bruker-Daltonics) to interrogate the NCBI non-redundant protein database using MASCOT software 2.3 (Matrix Science). Finally, two protein identification search programs were used to mine the sequences based on EST data; and as BLAST analysis, conducted in Research Support centers of the Center for Biological Research (CSIC, Madrid). Mass spectrometry (MS) analysis to determine the molecular mass of the purified proteins was carried out on a Bruker Reflex II Matrix-assisted laser-desorption ionization time-of-flight mass spectrometer (Bruker-Franzen Analytik, Bremen, Germany). The equipment was externally calibrated employing singly, doubly, and triply charged from cytochrome c (12 360 Da) or bovine serum albumin (66 430 Da).

ELISA test

Polystyrene 96-well plates (Costar, Fisher scientific, Madrid) were used for these assays. Each well was coated with 0.2 µg of purified protein in 100 µL of PBS and incubated overnight at 4 °C. Then, wells were blocked with PBS containing 3% non-fat dry milk and

0.1% Tween 20 at room temperature for 1 h. Plates were washed 3 times for 10 min with PBS 0.1% Tween 20 buffer and incubated with individual sera from anaphylactic patients (dilution 1:5 (v/v) in PBS 3% non-fat dry milk/ 0.1% Tween 20) for 2 h. After washing three times for 10 min in PBS 0.1% Tween 20, plates were incubated with mouse anti-human IgE (1:5000 (v/v) for 1 h more and, afterwards, incubated with HRP-labeled rabbit anti-mouse IgG (1:2500 (v/v)) for 10 min. The detection system was based in *o*-phenylenediaminedihydrochloride (OPD) measured registering visible absorption at 492 nm.

Basophil Activation Test

One hundred milliliters of heparinized whole blood was incubated with 20 μ L of stimulation buffer (1 M HEPES buffer containing 0.78% NaCl (w/v); 0.037% KCl (w/v); 0.078% CaCl_2 (w/v); 0.033% MgCl_2 (w/v); 0.1% HSA (w/v); 10 μ L/mL IL-3 (1 mg/mL) and 1 μ L of monoclonal antibody CCR3-APC (1 mg/mL) (BioLegend INC, San Diego, USA) for 10 min at 37 °C. Then, 100 μ L of the above mentioned proteins in Skin prick tests section (purified tomato seed nsLTP, Pru p 3, Ara h 9, and Hel a 3) and extracts (whole tomato and seeds) were added at different concentrations following a ten-fold dilution pattern (0.1-0.00001 μ g/mL) and incubated for 30 min at 37 °C. PBS and anti-human IgE (0.5 mg/mL) (BD Biosciences, NJ, USA), 100 μ L per well, were used as a negative and positive controls, respectively. After 5 min on ice, to stop the degranulation process, samples were incubated with monoclonal antibodies anti-CD 203c-PE and CD63 FITC (BioLegend) for 15-20 min at 4 °C. Finally, lysis solution (BD Biosciences) was used for red cells disruption. Cells were analyzed using a FACSCalibur flow cytometer (BD Biosciences), acquiring at least 500 basophils per sample. Results are presented as the percentage of activated basophils (CD63+CD203c+CCR3+). Moreover, the basophil sensitivity to the different allergens tested was compared using the CDsens which was calculated as the inverted value for LC_{50} , being this value the lowest allergen concentration giving 50% of maximum CD63% up-regulation in a dose response curve, multiplied by 100, as described [13].

Sequence determination and predictive analyses

The amino acid composition was determined using the corresponding DNA sequences. Site prediction of signal peptide processing in each of the allergens was conducted with the

prediction program SignalP-4.1. Secondary structure content prediction was performed using the PSIPRED computer program, according to the method described by Chou and Fasman [24].

Results

Clinical features of patients

Tomato-allergic patients (N=22) included in this study had the clinical features summarized in Table 1. Their average age of the patients was 33.5 ± 9.8 years showing a strong predominance of female individuals (95%). Clinical symptoms can be described as 11 patients (50%) showing OAS while the other 11 displayed systemic reactions. The most frequent of these systemic reactions were anaphylaxis in 32% and urticaria in 18% of the patients' sample. In addition, most of them showed positive SPT with other edible plants belonging to the Rosaceae family (88.9%), different nuts (83.3%), or other fruits of the latex-fruit syndrome (61.1%). Only one patient (5.5%) was also allergic to olive pollen (*Olea europaea*) and one more was exclusively allergic to tomato. The results of sIgE to tomato, although with low levels, showed a 62% of cases and did not correlate with the clinical symptoms of the patients.

Detection of IgE-reactive proteins in tomato seeds

Patients showed positive SPT with whole tomato in 95.5% of cases, with tomato seed extracts in 68% and with the tomato seed nsLTPs in 50% (Table 1). Controls did not show any positive result in SPT, even at the highest concentrations used.

In immunoblotting assays, IgE binding to reactive proteins from tomato seeds extract under reducing conditions showed different patterns of IgE recognition (Figure 1). Bands of around 12 kDa were observed in 5 out of 7 patients (71.4%) with anaphylaxis whereas they were only visible in one patient (9%) with mild symptoms. On the other hand, only IgE reactive bands corresponding to molecular masses between 30-50 kDa could be observed in patients with OAS.

Identification and structural characterization of two nsLTPs in tomato seeds

The IgE-reactive proteins from tomato seeds were fractioned by HPLC using a C-18 reverse phase column. The eluted proteins were quantified by BCA and visualized by means of SDS-PAGE. Dot blot analysis of the different fractions obtained was also performed, using a pool of sera from patients allergic to tomato with anaphylaxis (Figure 2A). This fraction correspond to a 30 kDa protein when the SDS-PAGE was carried out under non reducing conditions, altogether with a much fainter corresponding to a 10 kDa molecular mass. Interestingly, samples previously treated with β ME yielded only two bands of around 12 and 10 kDa (Figure 2B). The yield obtained was 139 mg for each gram of tomato seed extract.

These two IgE-reactive proteins were subjected to SDS-PAGE in the presence of β ME and were individually extracted from the gel and digested with trypsin. The resulting peptides were analysed by MALDI-TOF-TOF, subjected to fragmentation and MS analysis. The identified peptide sequences from each protein are shown in the MS-profiles (Figure 3A). Comparison of these sequences with those ones contained in different databases showed coincidence with two basic proteins from tomato seeds (nsLTP class 1 (XP_004229753.1) and nsLTP class 2 (P86417)). The peptide coverage is 56.2% and 70.8%, respectively.

MS analysis of the mixture of both nsLTPs in the presence of β ME purified from HPLC, render two peaks corresponding to polypeptides with molecular masses of 9416.5 and 7023.7 Da, which match well with the theoretical masses of the predicted proteins from the GenBank databases. The expected pI values are 8.49 and 8.59, respectively. The former one, showing the higher molecular mass, had not been described as an allergen and therefore the name Sola l 7 was assigned by the World Health Organization/International Union of Immunological Societies (WHO/IUIS) Allergen Nomenclature Sub-Committee (www.allergen.org). The other smaller protein is a recently recognized tomato allergen known as Sola l 6. The amino acid identity degree of both proteins with that of the tomato peel allergenic nsLTP Sola l 3 was of only 22% and 38%, with Sola l 6 and Sola l 7, respectively (Figure 3B), reaching a 61% and 35% of amino acid similarity degree. The amino acid identity and similarity values between the now described tomato seeds allergens Sola l 6 and Sola l 7 were 16% and 32% respectively, according to the program used.

IgE-binding capacity of Sola l 6 and Sola l 7 from tomato seeds

The IgE-binding capacity of both Sola l 6 and Sola l 7 was assayed by ELISA and immunoblotting. This latter assay was performed after fractionating them by SDS-PAGE made in reducing conditions (Figure 4). All the sera from patients showing severe symptoms were positive against any of the proteins mixture (Sola l 6 and Sola l 7) according to the ELISA results recognizing the band of lower electrophoretic mobility, corresponding to Sola l 7. However, maybe by the less IgE recruitment by Sola l 6 only two sera were clearly positive against the band corresponding to this allergen. Furthermore, five out of the seven patients with anaphylaxis (71.4%) were positive to Sola l 7 in immunoblotting.

Basophil Activation Test

The allergenic capacity of the purified mixture of Sola l 6 and Sola l 7 obtained from tomato seeds was finally evaluated *ex vivo* by performing BAT in 12 tomato allergic patients displaying different clinical symptoms (4 with anaphylaxis (cases 1, 3, 4 and 6), 4 with urticaria (cases 8-11) and 4 with OAS (cases 12, 14, 16 and 18) (Figure 5). Data showed that although patients with severe, moderate and mild symptoms had high basophil reactivity with tomato extract, only those with anaphylaxis presented a higher level of basophil activation with both the extract and the purified mixture of nsLTP from tomato seeds. These patients showed also reactivity and sensitivity to nsLTP from other vegetables, being very high for peach (Pru p 3) and peanut (Ara h 9), with CD-sens values of $118.28 \cdot 10^3$ and $170.29 \cdot 10^4$ respectively, but much lower for sunflower seed (Hel a 3; CD-sens value of $32.03 \cdot 10^3$). It is also remarkable that the basophil sensitivity to tomato seed nsLTPs (Sola l 6 and Sola l 7), measured in terms of CD-sens values, was one thousand fold higher in patients with anaphylaxis ($235.3 \cdot 10^3$) when compared to urticaria (249.04) or OAS patients (202.09).

Sola l 6 and Sola l 7 from tomato seeds show *in vitro* IgE cross-reactivity

A pool made with seven sera from patients showing anaphylaxis symptoms was used to perform *in vitro* IgE-inhibition immunoblotting experiments after electrophoretic fractionation of the purified mixture of allergens. Extracts, from different parts of tomato fruit, such as placenta, pulp and peel, and, from other foods, such as peanut, seeds from sunflower, kiwi, and mustard, or peach and apple were used as inhibitors (Figure 6). Not

surprisingly, IgE binding was completely inhibited with the tomato seeds extract. Placenta tissue produced inhibition of about 82%, while, neither pulp nor peel extracts were able to significantly inhibit IgE binding. On the other hand, peanut, peach and apple almost completely inhibited IgE binding to the purified proteins, suggesting the existence in these extracts of proteins displaying strong cross-reactivity. Finally, whereas sunflower seeds extract only showed partial inhibition, kiwi and mustard seeds were completely non-reactive.

Discussion

Studies on tomato allergy have been focussed in patients sensitized to pulp and peel of the fruit [3, 25]. Tomato is one of the most consumed and cultured food worldwide an increase that has grown together with the allergic incidence [26]. The prevalence of sensitization to this food ranges from 1.5% to 25.0% depending on the population studied. Patients are often diagnosed because they present clinical symptoms but paradoxically in many instances they give negative results in conventional diagnostic skin tests [4]. This low sensitivity of the diagnostic tests could be due to a low concentration of relevant allergenic proteins in the whole extracts employed. Moreover, could be also attributed to the different content of allergenic proteins depending on the tomato varieties studied [27], the ripening degree or the different distribution of allergens in the tissues of tomato fruit. In this context, it has also to be considered the additional problem, which represent some of the new ways of preparing meals, which involve blending all components together, being then more difficult to discard those specific components that cause allergy. In summary, all these aspects enhance the importance of further analyzing all allergenic proteins of all allergenic proteins in order to improve the diagnostic tests for evaluating these allergic patients. Currently, six tomato allergenic proteins have been identified and described in pulp and peel [25] being Sola 1 3 associated with clinical symptoms [28] and, additional ones have been also detected in seeds [2]. Accordingly, the aim of the present study was the identification of new allergens with low representation in the fruit but concentrated in the seeds of tomato. The initial hypothesis was that these potential new allergens could be relevant for the tomato-allergic diagnosis associated to severe symptoms. The important role of underrepresented allergens in allergy manifestations has been reported before with other foods [8, 9]. These authors demonstrated the existence of allergenic LTP and storage proteins in kiwifruit seeds, for example. An

1 observation which, again, suggests that in fruits allergenic reactions might be also caused by
2 proteins located at the seeds.

3 In the study reported now, sera from the patients with anaphylaxis were tested in
4 immunoblotting with homemade tomato seed extracts showing two highly frequent IgE-
5 reactive protein bands of 12 and 10 kDa. These proteins were identified by proteomic tools as
6 two nsLTP, Sola l 7 and Sola l 6, belonging to classes 1 and 2, respectively. Around 50% of
7 all the tomato allergic patients included in this study were positive to both allergens in a SPT,
8 whereas 71.4% of those ones with anaphylaxis showed positive responses in immunoblotting
9 and BAT. Interestingly, in patients with urticaria, only none or low responses to these
10 allergenic proteins were observed in equivalent experiments in these *in vitro* tests.

11 The three nsLTP identified in tomato tissues have significant differences in primary structure
12 because that one described in pulp and peel, called Sola l 3, displays a very low amino acid
13 identity degree reaching barely 38% and 22%, with those present in seeds, Sola l 7 and Sola l
14 6, respectively. Sola l 7 is the most similar to other members of this protein family with Act d
15 10 (53%), Pru p 3 (41%) and Ara h 9 (51%). However, out of the three tomato nsLTPs, Sola l
16 6 is the most different protein from other nsLTP members, with an amino acid identity of
17 18%, 25%, and 26% with those of kiwi, peach and peanut, respectively. The availability of
18 two allergenic nsLTPs, Sola l 6 and Sola l 7, from tomato seeds together with Sola l 3
19 identified in pulp and peel, might contribute to improve the diagnostic sensitivity, particularly
20 considering their allergenic contribution in the severity of symptoms of certain patients
21 allergic to this vegetable food. In fact, results from BAT show a very high reactivity and
22 sensitivity to these nsLTP in patients with anaphylactic reactions compared to those with
23 moderate and mild symptoms, a very important observation regarding diagnosis and
24 treatment. Indeed, all these results further support the strong association of food allergy with
25 the sensitization to nsLTPs found in Southern Europe [29].

26 The involvement of the allergenic nsLTPs from tomato fruit in cross-reactivity processes with
27 other vegetable foods such as fruits from the *Rosaceae* family or even a material like latex,
28 adds more complexity to the diagnosis of this food allergy [30, 31]. nsLTPs are indeed
29 considered important panallergens [31-33]. In this paper we analyzed this *in vitro*
30 phenomenon between the nsLTPs purified of tomato and different extracts as foodstuffs, such
31 as peach, nuts, or seeds from mustard, sunflower and kiwi with the studied allergic
32 population. We chose these extracts because, first most of the patients had SPT positive to
33 them and second, nsLTP proteins have been identified as major allergens in these sources.

The results of the inhibition of IgE binding to the purified tomato seeds nsLTPs demonstrated the existence of cross-reactivity with sunflower seeds, peach, apple and peanut but not with kiwi or mustard seeds. Considering that the currently known tomato allergens have been exclusively described in pulp or peel, together with the fact that seeds are commonly ingested with the pulp but correspond to a small percentage of the whole fruit, we propose that Sola l 6 and Sola l 7 are key allergens related with severe symptoms, which may be masked in the context of tomato IgE sensitization. This present identification is undoubtedly going to clarify the diagnosis of tomato allergy.

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Authors contribution

The Project outline was drafted by BM, VM, MC, RR.

The experiments were performed by M-P L, GM, MC.

Data analysis and patients recruitments G-A M, GF, B-GN, TMJ, VM.

All the authors (VM, M-P L, GM, BM, MC, RR, G-A M, GF, B-GN, TMJ) provided input to drafting the article and revising it critically for important intellectual content and gave final approval of the versión to be published.

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Conflict of interest statement

The authors have declared no conflict of interest

Figures

Figure 1. Tomato seed extract and patient sera recognition: SDS-PAGE of tomato seeds extract and immunoblotting detecting IgE reactivity of individual sera from patients allergic to tomato with anaphylaxis 1-7; urticaria 8-11; OAS 12-22. A non atopic individual was used as negative control (C). Molecular masses of standard proteins are indicated.

Figure 2. Purification of Sola l 6 and Sola l 7: (A) reverse phase-HPLC profile of the tomato seeds extract using an acetonitrile gradient (0-60%) Insets: (A). Dot blot of the fractions stained with a pool of six sera allergic to tomato with anaphylaxis. (B) SDS-PAGE and CBS of the fraction n° 7 without and with β ME.

Figure 3. Identification of two new allergens: (A) MS-profile of the indicated protein bands. Inset, SDS-PAGE of tomato seeds purified nsLTPs stained with CBS under reducing conditions and a pool of six sera. Framed sequences correspond to the peptides identified by MS/MS analysis. (B) Amino acid sequence alignment of Sola l 3 nsLTP (UNIPROT accession number P93224) with peptides identified by MS/MS analysis. The two nsLTPs identified in tomato seeds are also included. % of amino acid identity and similarity is shown in the figure. Peptide sequences matching to peptides obtained by MS are boxed in gray.

Figure 4. Recognition of purified allergens: Specific IgE-binding ability to Sola l 6 and Sola l 7 (1 μ g) of 7 individual sera from patients allergic to tomato with anaphylaxis. C is a serum from a non-atopic subject.

Figure 5. Bashophil Activation Test: Graphs represent mean and SD of %CD63 cells from 12 representative cases of patients allergic to tomato with different clinical symptoms, Anaphylaxis (N=4), Urticaria (N=4) and OAS (N=4) and Controls (N=6).

Figure 6. Inhibition assay: Inhibition of the IgE binding to purified Sola l 6 and Sola l 7 by the indicated protein extracts (300 µg) as inhibitors using a pool of sera (1:5) from tomato allergic patients with anaphylaxis. PBS was used as negative control of inhibition. Percentages of inhibition obtained after densitometer of the blots are shown at the bottom.

Table 1. Demographic and clinical characteristics of the patients

Nº Pat	Age (Years)	Sex	Clinical Symptoms	Skin test											slgE tomato (kU/L)
				Tomato*	Nuts & sunflower seeds*	Rosaceae*	Fruit-latex*	Olea pollen*	Tomato pulp	Tomato seed	Sola l 6/7	Pru p 3	Ara h 9	Hel a 3	
1	37	F	Anaph	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	2.16
2	32	F	Anaph	Pos	Pos	Pos	Neg	Neg	Pos	Neg	Neg	Pos	Pos	Neg	1.66
3	58	M	Anaph	Pos	Pos	Pos	Pos	Neg	Neg	Pos	Pos	Neg	Pos	Pos	0.02
4	40	F	Anaph	Pos	Pos	Pos	Pos	Neg	Pos	Pos	Pos	Pos	Pos	Pos	19.8
5	38	F	Anaph	Pos	Neg	Neg	Neg	Neg	Pos	Pos	Pos	Neg	Neg	Neg	0.04
6	39	F	Anaph	Pos	Pos	Pos	Neg	Neg	Pos	Pos	Pos	Pos	Pos	Pos	0.1
7	50	F	Anaph	Pos	Pos	Pos	Pos	ND	Pos	Neg	Neg	Neg	Neg	Neg	1.41
8	20	F	Urt	Neg	Neg	Pos	Pos	Neg	Neg	Pos	Pos	Pos	Pos	Neg	0.04
9	23	F	Urt	Pos	Pos	Pos	Neg	Neg	Neg	Pos	Pos	Pos	Pos	Pos	5.12
10	25	F	Urt	Pos	Neg	Pos	Pos	Neg	Pos	Neg	Neg	Pos	Neg	Neg	0.27
11	44	F	Urt	Pos	ND	ND	ND	ND	Pos	Pos	Pos	Neg	Neg	Neg	0.1
12	27	F	OAS	Pos	Pos	Pos	Pos	Neg	Pos	Pos	Pos	Pos	Pos	Pos	0.97
13	28	F	OAS	Pos	Pos	Pos	Pos	Neg	Neg	Pos	Neg	Neg	Pos	Neg	0.2
14	35	F	OAS	Pos	Pos	Pos	Pos	Neg	Neg	Neg	Neg	Neg	Pos	Neg	0.83
15	23	F	OAS	Pos	Pos	Pos	Neg	Neg	Neg	Pos	Pos	Pos	Pos	Pos	2.5
16	30	F	OAS	Pos	Pos	Pos	Pos	Neg	Neg	Pos	Neg	Pos	Pos	Neg	8.32
17	28	F	OAS	Pos	Pos	Pos	Neg	Neg	Pos	Neg	Neg	Neg	Pos	Neg	5.67
18	26	F	OAS	Pos	Pos	Neg	Neg	Neg	Pos	Pos	Pos	Neg	Pos	Neg	N/D
19	34	F	OAS	Pos	Pos	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Neg	Neg	0.87
20	40	F	OAS	Pos	ND	ND	ND	ND	Neg	Pos	Neg	Neg	Pos	Neg	0.18
21	20	F	OAS	Pos	ND	ND	ND	ND	Neg	Pos	Neg	Neg	Neg	Neg	0.55
22	40	F	OAS	Pos	ND	ND	ND	ND	Neg	Neg	Neg	Neg	Neg	Neg	0.36

F: Female; M: Male; Anaph: Anaphylaxis; Urt: Urticaria; OAS: Oral allergic syndrome; *: Commercial extracts; Nuts include: hazelnut, walnut, peanut, sunflower seed and almond; Rosaceae include: peach, plum, apple and apricot; Fruit-latex include: banana, kiwifruit, avocado, fig and chestnut; Pos: Positive; Neg: Negative; ND: Not done;

Figure 1

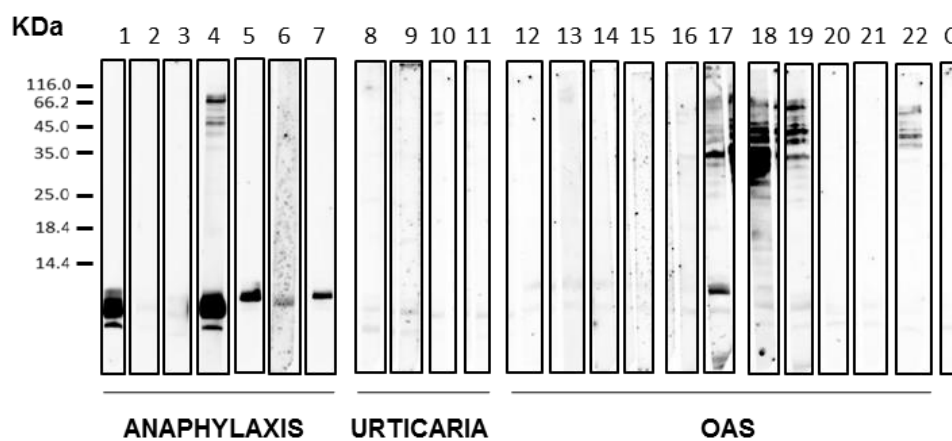


Figure 2

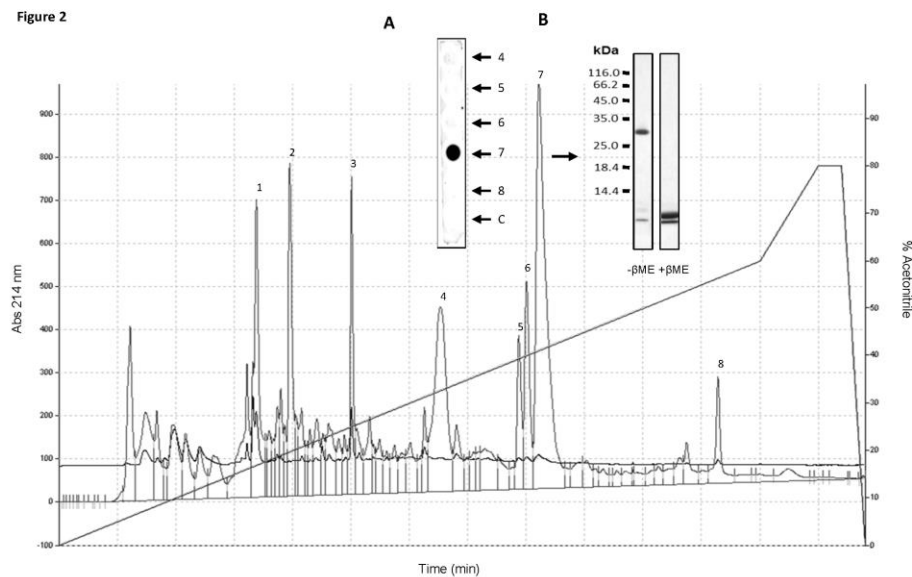
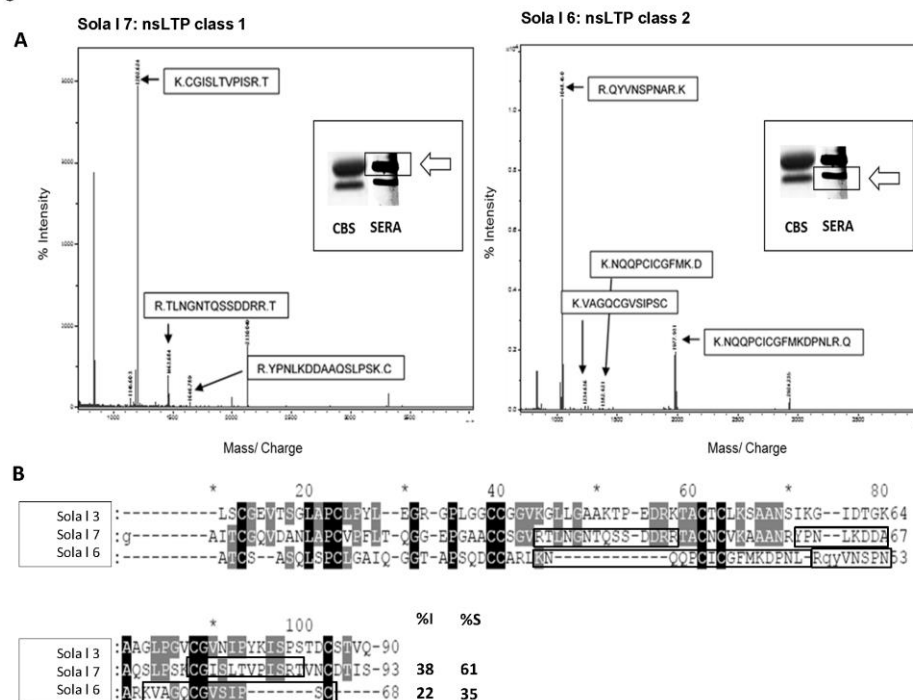


Figure 3



1

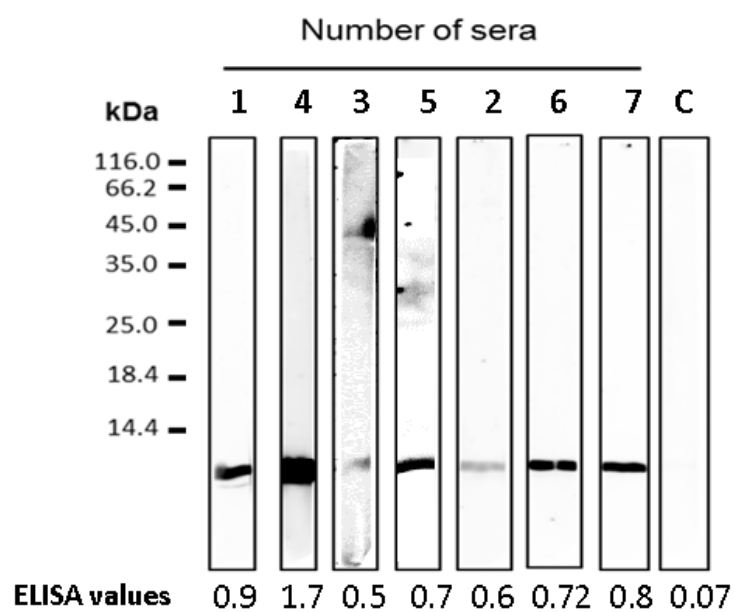
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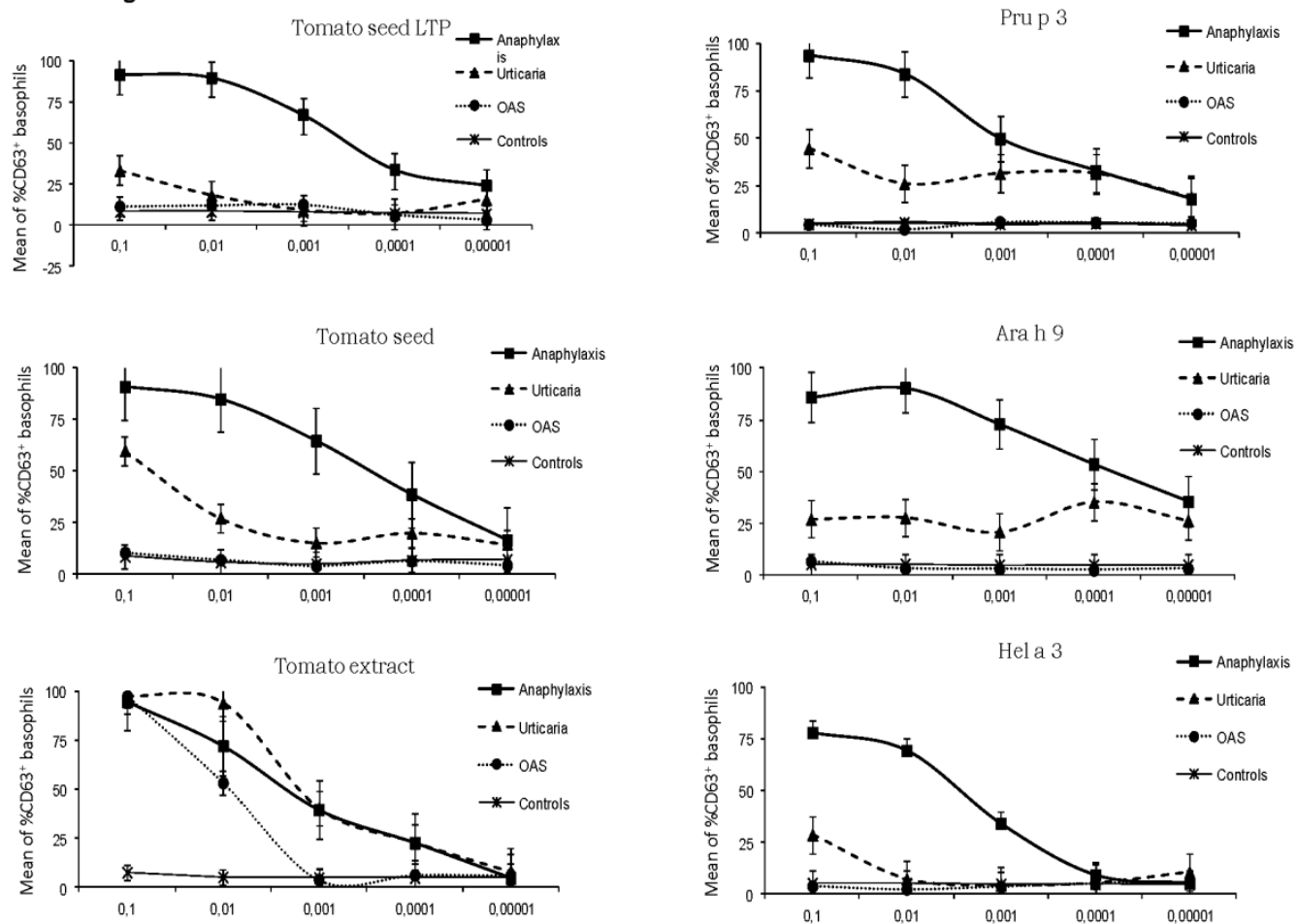
Figure 5

Figure 6

