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Review

Plant non-specific lipid transfer proteins: An interface between plant defence and human allergy

G. Salcedo^{a,*}, R. Sánchez-Monge^a, D. Barber^b, A. Díaz-Perales^a^a Unidad de Bioquímica, Departamento de Biotecnología, E.T.S. Ingenieros Agrónomos, UPM, Ciudad Universitaria, 28040-Madrid, Spain^b Departamento de I+D, ALK-Abelló, Madrid, Spain

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Abstract

Plant non-specific LTPs (lipid transfer proteins) form a protein family of basic polypeptides of 9 kDa ubiquitously distributed throughout the plant kingdom. The members of this family are located extracellularly, usually associated with plant cell walls, and possess a broad lipid-binding specificity closely related to their three-dimensional structure. The nsLTP fold is characterized by a compact domain composed of 4 α -helices, firmly held by a network of 4 conserved disulphide bridges. This fold presents a large internal tunnel-like cavity, which can accommodate different types of lipids. nsLTPs are involved in plant defence mechanisms against phytopathogenic bacteria and fungi, and, possibly, in the assembly of hydrophobic protective layers of surface polymers, such as cutin. In addition, several members of the nsLTP family have been identified as relevant allergens in plant foods and pollens. Their high resistance to both heat treatment and digestive proteolytic attack has been related with the induction by these allergens of severe symptoms in many patients. Therefore, they are probably primary sensitizers by the oral route. nsLTP sensitization shows an unexpected pattern throughout Europe, with a high prevalence in the Mediterranean area, but a low incidence in Northern and Central European countries.

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Keywords: Lipid transfer protein; Plant defence; Plant allergen; Food allergy; Panallergen family**1. Introduction**

A large non-specific lipid transfer protein (LTP) family, comprising over 100 potential members from up to 50 different species, has been described [1,2] since the discovery of these proteins 30 years ago [3]. Furthermore, more than 150 DNA sequences encoding putative related proteins, designated as nsLTP-like, have been also located in up to 45 plant species [2]. The possible functions of nsLTPs in plants have been extensively debated over the last years, but their role in plant defence mechanisms against phytopathogens (bacteria and fungi) seems to be well-established now [1,4,5]. Moreover, an unexpected role of nsLTPs from *Rosaceae* fruits in IgE-mediated allergic reactions was uncovered in 1999 after studying adult patients from the Mediterranean area with allergy to these fruits [6,7]. The large clinical evidence accumulated afterwards has

allowed to define nsLTPs as a relevant panallergen family of both plant foods and pollens [8–11]. Interestingly, other plant defence proteins, such as chitinases, thaumatins and PR (pathogenesis-related)-10 proteins, have also been identified as plant allergens [12,13], thus sharing with nsLTPs a role in plant protection and human disease.

2. The 9 kDa nsLTP family

Two main types of plant nsLTPs have been described: nsLTP1 of 9 kDa and nsLTP2 of 7 kDa [14,15]. In addition, an increasing number of related nsLTP-like proteins are also being identified [2]. The nsLTP1 group will be the focus of this review. These proteins, together with other plant protein families (cereal prolamins, cereal α -amylase/trypsin inhibitors, 2S albumins, puroindolines, etc.) constitutes the prolamin superfamily [2,13].

* Corresponding author. Tel.: +34 91 336 56 96; fax: +34 91 336 57 57.

E-mail address: gabriel.salcedo@upm.es (G. Salcedo).

The 9 kDa nsLTP family is widely distributed throughout the plant kingdom. It comprises basic polypeptides (pI 8.5–10) with 90–95 amino acid residues, lacking Trp among them. Eight conserved cysteine residues form a network of 4 disulphide bridges, which are essential to maintain the nsLTP fold and, therefore, the lipid-binding and allergenic properties of these proteins [1,2,11,14,16]. Additionally, 2 consensus sequences, T/SXXDR/K and PYXIS, have been located around residues 43–47 and 81–85, respectively [14]. The 9 kDa nsLTPs are encoded by a divergent multigene family, dispersed in different chromosomes, in most plant species studied. Thus, at least 10 genes (together with 60 more genes encoding potential nsLTP-like proteins) have been identified in *Arabidopsis thaliana* [2,17], as well as more than 10 genes in rice (*Oryza sativa*) [18] and 15 genes in hexaploid wheat (*Triticum aestivum*) [15]. Differential and complex expression patterns have been found for the different genes, characterized by a tissue and developmental specificity and also modulated by biotic and abiotic stress [1,4,15,18].

2.1. Extracellular accumulation and tissue distribution of plant nsLTPs

It is now well known that the nsLTPs are synthesized as precursors with a typical N-terminal signal peptide of 20–25 amino acids and the pre-proteins enter the secretory pathway. The mature polypeptides are secreted outside the cell, and are usually associated with cell walls [1,4,16]. This extracellular location is consistent with the secretion of nsLTPs into aleurone and embryogenic cell cultures [19,20]. In contrast, their intracellular location, such as in castor bean glyoxisomes or *Vigna unguiculata* protein storage vacuoles, has been exceptionally proposed [21,22].

The nsLTP family is ubiquitously expressed throughout the plant and has been found in several organs, such as seeds, siliques, leaves, stems, fruits, flowers, and, most rarely, roots. The highest expression levels have been found in peripheral cell layers surrounding aerial organs, associated with cell wall and cuticle of epidermal tissues [1,4]. This expression pattern supports the role of nsLTPs in both plant defence against microorganisms invading from the outside and deposition of extracellular cutin (see below). Furthermore, relevant clinical implications can be derived from the accumulation of allergenic nsLTPs in the external tissues of plant foods, such as the peel of fruits [23,24].

2.2. The nsLTP fold and its lipid-binding capacity

The non-specific lipid transfer proteins (nsLTP) were originally so named by their ability to bind and enhance, *in vitro*, the transfer of different types of lipid molecules between membranes [1,14]. However, an *in vivo* role of these proteins in the cell plant intracellular (cytoplasmic) trafficking of membrane lipids seems now unlikely, considering the extracellular localization of nsLTPs mentioned above. The 9 kDa members of this family show a broad lipid-binding specificity, including fatty acids, fatty acyl-CoA, phospholipids, glycolipids, hydro-

xylated fatty acid and prostaglandin B₂ [1,14,25,26]. Variation of the binding specificity has been shown among different nsLTP members [27,28]. The lipid-binding capacity of these proteins is closely related to the peculiar characteristics of the nsLTP fold. Several 3-D structures of 9 kDa plant nsLTPs, both free and forming complexes with different lipidic ligands, have been determined by either X-ray crystallography or nuclear magnetic resonance. Thus, the structures of wheat [25,29–31], barley [32–34], maize [35–37], rice [38–40], tobacco [41], mung bean [42] and peach [43] nsLTPs have been resolved. All of them show the typical nsLTP fold, characterized by an α -helical compact domain composed by 4 α -helices (H1–H4) connected by short loops (L1–L3) and a non-structured C-terminal tail. The fold is strongly held by a network of 4 disulphide bridges (connecting Cys residues 3–50, 13–27, 28–73, and 48–87 in peach nsLTP corresponding to the allergen Pru p 3 shown in Fig. 1), which is essential for lipid-binding [44]. Additionally, a large number of intramolecular H-bonds contribute to the stabilization of the 3-D protein structure. Besides its unusual stability, the main feature of nsLTP folding is the presence of a large internal tunnel-like cavity following the long axis of the molecule. The surface of this cavity is coated with the side chain of hydrophobic residues included in the four helices (H1–H4) and the C-terminal tail (for example, residues Val 6, Met 10, Leu 14, Tyr 16, Val 17 in H1; Val 31, Leu 34 in H2; Val 47, Leu 51, Ile 54 in H3; Ala 66, Ile 69 in H4; and Tyr 79, Ile 81 in C-terminus of the barley nsLTP; see ref. 33 and Fig. 1). Different lipid types, such as those mentioned above, can be accommodated in the cavity, which binds their hydrophobic parts (i.e. C10–C18 tails of fatty acids). This internal cavity exhibits a high plasticity, adapting and increasing its volume (from around 200 to 750 Å³) upon lipid-binding [14,31,34,37,44]. The expansion of the cavity seems to depend on the size of the bound ligand [33,34,37]. This structural plasticity and non-specific van der Waals interactions with the hydrophobic ligand tail can contribute to the non-specificity of nsLTP regarding lipid-binding capacity [37]. However, such capacity varies among members of the family. For instance, tobacco nsLTP1-1 binds only one fatty acid or phospholipids, whereas wheat or maize nsLTPs can interact with one or two [28,31,40]. Other plant proteins with allergenic activity, such as the hydrophobic protein corresponding to the soybean allergen Gly m 1, the 2S seed storage proteins and the α -amylase/trypsin inhibitors from cereal flours, display 3-D structures close to the nsLTP fold, but do not show a similar internal lipid-binding cavity [45–47].

2.3. Proposed functions in plants

Since the initially suggested role of nsLTPs in the cytoplasmatic trafficking of membrane lipids was ruled out, the actual *in vivo* function of this plant protein family has been a matter of debate. Nevertheless, at least its role in plant defence mechanisms is now well established [4,48].

Several lines of evidence point to the role of nsLTPs in the protective mechanisms developed by plants against the attack of bacteria, fungi and, possibly, viruses. This has led to the

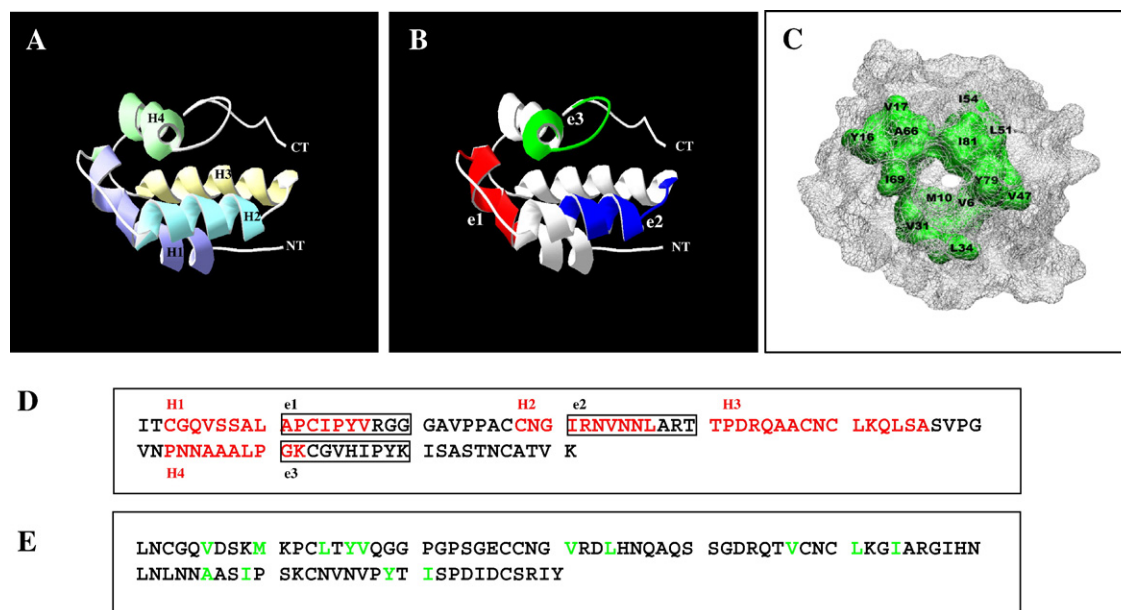


Fig. 1. 3-D structure of the nsLTP and major allergen from peach fruit, showing the nsLTP-fold (A) and its main sequential human IgE-binding regions (epitopes, e1 to e3) (B). 3-D structure of barley nsLTP, highlighting the lipid-binding cavity (C). Amino acid sequence of peach nsLTP indicating the positions of the four α -helices (H1–H4; red residues) and the IgE epitopes (e1–e3; boxed residues) (D). Amino acid sequence of barley nsLTP, showing the residues located on the surface of the hydrophobic lipid-binding cavity (marked in green) (E).

classification of nsLTPs as pathogenesis-related (PR) proteins, which are included in the PR-14 family [5]. The evidence now available can be summarized as follows: (a) the tissue distribution and extracellular localization discussed above are compatible with the existence of a defensive-nsLTP shield in the plant surfaces susceptible of pathogen attack [4]. (b) nsLTPs from different plant species show *in vitro* activity against phytopathogenic bacteria and fungi [4,48–52]. The relative activities vary between strains and species of pathogen, indicating some degree of specificity in each nsLTP. (c) Barley and sunflower nsLTP induce a weak leakage of model membranes (liposomes) [53,54] and what is more interesting is that the later protein provokes permeabilization of intact spores of the pathogenic fungus *Fusarium solani* in a dose-dependent manner [54]. (d) The transgenic expression of barley nsLTP in both tobacco and *Arabidopsis* plants enhances the tolerance to a bacterial pathogen (*Pseudomonas syringae*) [55]. Furthermore, nsLTP (and other defence proteins) accumulate during grape fruit ripening as a developmentally regulated defence response against phytopathogens [56]. (e) The expression of several *Ltp* genes respond to bacterial and fungal attacks, frequently with complex and differential expression patterns [1,4,16,57]. Induction of *Ltp* gene expression by viral infection has also been reported [58,59]. In addition, nsLTPs expressed in grape cell suspensions are induced in response to various defence-related signal molecules, such as fungal elicitors [60]. (f) An nsLTP from *Arabidopsis*, probably interacting with a lipid-derived compound (oxylipins), seems to promote long-distance signalling throughout the plant, during the so-called systemic-acquired resistance (SAR) [61,62]. Novel evidence recently published on nsLTPs as potential signal molecules represents a further step in their link

to plant defence. (g) A wheat nsLTP binds to the receptors of fungal elicitors in host plant membranes, and behaves as an elicitor antagonist [63]. Elicitins are sterol-binding proteins of around 10 kDa, secreted by oomycetes fungal pathogens. Elicitor–receptor interaction triggers a hypersensitive response and non-specific systemic resistance in the host plant, which are mediated by a cascade of signalling pathways. Competition between endogenous nsLTPs and elicitors on the receptors can modulate such responses [64]. (h) Finally, an *Arabidopsis* nsLTP has been identified as a calcium-independent calmodulin-binding protein, suggesting that calmodulin signalling could mediate some nsLTP functions in plants [65].

The role of nsLTPs in plant defence is compatible with other potential functions that have been proposed for this protein family. Particularly, a second mechanism related with the strengthening of structural barriers against pathogen attack, in line with the extracellular accumulation of nsLTPs in outer tissues of plant organs, has been outlined. nsLTPs would be involved in the assembly of hydrophobic protective layers of surface polymers, such as cutin [14,16,20], transporting cutin-monomers during cuticle formation. This role is supported by their capacity to bind hydroxyl-derivates of fatty acids [26].

In addition, abiotic stress factors, such as drought, cold and high salinity, have been described to upregulate members of the nsLTP family in some plant species [1,16,66–68]. Stabilization of membranes, cuticle deposition and/or changes in cell wall organization have been claimed as their putative roles in the responses to these stress factors [14,66,68]. In addition, nsLTPs have a potential role in plant growth and development, including embryogenesis [1,69], germination [70], and pollen–pistil interaction [71]. The ability of tobacco

and wheat nsLTPs to enhance cell wall extension is a new insight in this context [72].

3. nsLTPs as plant allergens

3.1. From peach allergens to plant food and pollen panallergens

IgE-mediated food allergy is most commonly triggered by plant foods, particularly fruits of the *Rosaceae* family (apple, peach), in the adult population [73–75]. The perception of allergy to these fruits (mainly apple) was ruled until the last decade by its association with birch pollinosis, which is frequent in Central and North European areas where Fagales trees are abundant. Patients usually show mild symptoms restricted to the oropharyngeal cavity, known as Oral Allergy Syndrome, and seem to be primary sensitized by pollen allergens [73,74,76]. The subsequent reaction to plant foods, such as apple, is based on the presence of homologous allergens in both plant allergenic sources: members of the PR-10 protein family related to the major birch pollen allergen Bet v 1, plant profilins related to birch Bet v 2, and complex N-linked glycans, designated as carbohydrate cross-reactive determinants [77–79]. The population allergic to *Rosaceae* fruits, mostly peach, in Mediterranean countries is nearly unexposed to Fagales pollen and displays a different clinical and sensitization profile. Severe systemic manifestations are frequently found, and a relevant number of patients suffer fruit allergy without linked pollinosis [80–83]. An uncharacterized IgE-binding band of approximately 10–13 kDa was initially located in crude extracts of peach and other *Rosaceae* fruits (apple, cherry, plum) as early as 1994, using sera from patients sensitized to fruit but not to pollen [84,85]. The reactive component was preferentially accumulated in fruit peels [80,84] and seemed to be different from those allergens associated with birch pollinosis. The identification of the 10–13 kDa IgE-binding component from peach and apple as members of the nsLTP family was independently published in 1999 by Spanish and Italian groups [6,7,86]. A major allergen of around 9 kDa was isolated from each species and designated Pru p 3 (peach, *Prunus persica*) and Mal d 3 (apple, *Malus domestica*), according to the guidelines of the I. U. I. S. Allergen Nomenclature Sub-Committee.

Since then, nsLTPs have been on the rise in relation to human allergy, and the work of several clinical and biochemical research groups has confirmed and extended their role in allergic reactions to plant foods and even pollens. Thus, allergenic members of the nsLTP-family have been identified and at least partially characterized in other *Rosaceae* fruits (apricot, plum, etc.), grape, citrus fruits, tomato, vegetables (asparagus, lettuce, etc.), nuts (hazelnut, walnut, etc.) and maize (see Table 1 for relevant references) [87–111]. Amino acid sequence identity between peach Pru p 3 and each of the other plant food allergen listed in Table 1 varies between 92% and 47% (Fig. 2), indicating their structural relationship. Besides these well-established and officially designated allergens, additional members of the family with potential

Table 1

Members of the nsLTP family identified as allergens in plant foods, latex and pollens

Plant species	Allergen	References
<i>PLANT FOODS</i>		
<i>Rosaceae</i> fruits		
Peach (<i>Prunus persica</i>)	Pru p 3	[6,7]
Apple (<i>Malus domestica</i>)	Mal d 3	[6,86]
Apricot (<i>Prunus armeniaca</i>)	Pru ar 3	[87]
Plum (<i>Prunus domestica</i>)	Pru d 3	[88]
Cherry (<i>Prunus avium</i>)	Pru av 3	[89]
Strawberry (<i>Fragaria ananassa</i>)	Fra a 3	[90]
Other fruits		
Grape (<i>Vitis vinifera</i>)	Vit v 1	[91]
Orange (<i>Citrus sinensis</i>)	Cit s 3	[92]
Lemon (<i>Citrus limon</i>)	Cit l 3	[92]
Tomato (<i>Lycopersicon esculentum</i>)	Lyc e 3	[93]
Vegetables		
Asparagus (<i>Asparagus officinalis</i>)	Aspa o 1	[94]
Lettuce (<i>Lactuca sativa</i>)	Lec s 1	[95]
Cabbage (<i>Brassica oleracea</i>)	Bra o 3	[96]
Nuts		
Hazelnut (<i>Corylus avellana</i>)	Cor a 8	[97]
Walnut (<i>Juglans regia</i>)	Jug r 3	[98]
Chestnut (<i>Castanea sativa</i>)	Cas s 8	[99]
Cereals		
Maize (<i>Zea mays</i>)	Zea m 14	[100]
<i>LATEX</i>		
Latex (<i>Hevea brasiliensis</i>)	Hev b 12	[101]
<i>POLLENS</i>		
Parietaria (<i>Parietaria judaica</i>)	Par j 1	[102,103]
	Par j 2	[104]
	(<i>Parietaria officinalis</i>) Par o 1	[105]
Ragweed (<i>Ambrosia artemisiifolia</i>)	Amb a 6	[106,107]
Olive (<i>Olea europaea</i>)	Ole e 7	[108]
Mugwort (<i>Artemisia vulgaris</i>)	Art v 3	[109,110]
Plane (<i>Platanus acerifolia</i>)	Pla a 3	[111]

Only those members included in the List of Allergens (International Union of Immunological Societies, Allergen Nomenclature Sub-Committee; searched on August 2006) are considered.

allergenic capacity have been isolated or detected in other foods and plant-derived beverages, such as pomegranate, onion, carrot, peanut, rice, spelt wheat, and beer [112–118]. Cross-reactivity among food nsLTP allergens has been described, even between members from botanically unrelated species [85,96,109,114,115,119]. However, both common and specific IgE epitopes seem to be present in the allergenic nsLTPs studied [109,110].

The widespread distribution of this protein family among plant species and organs explains its involvement in reactions elicited by non-food plant sources. Thus, Hev b 12, a 9 kDa nsLTP related to (65% of amino acid sequence identity), and immunologically cross-reactive with peach Pru p 3, has been described as a minor allergen of natural rubber latex [101].

The status of nsLTPs as pollen allergens (Table 1), a crucial matter regarding their potential routes of sensitization, is more complex. Taking into account their similarity and cross-reactivity with their putative homologues from plant foods, particularly Pru p 3, as well as their prevalence among the corresponding pollinic patients, three different types can be considered. Mugwort Art v 3

Allergen	Amino acid sequence	% Id
Pru p 3 (CAB96876)	ITCGQVSSSLAPCI ¹ EVVRGGGAVPFA-C ² CGN ³ IRNVN ⁴ LART ⁵ TPDR ⁶ AACNCLKQLSASVPGVNPNNAAAL ⁷ PKCGVSI ⁸ PY--KISASTNCATVK	
Mal d 3 (CAB96874)	ITCGQVSSSLAPCI ¹ GVVSGGAVPFA-C ² CGN ³ ITING ⁴ LART ⁵ ADRTACNCLKNLAGSISGVNPNNAAAGL ⁷ PKCGVNPV ⁸ Y--KISTSTNCATVK	81
Pru ar 3 (P81651)	ITCGQVSSSLAPCI ¹ GVVRGGGAVPFA-C ² CGN ³ IRNVN ⁴ LART ⁵ TPDR ⁶ TACNCLKQLSGSISGVNPNNAAAL ⁷ PKCGVNPV ⁸ Y--KISASTNCATVK	92
Pru d 3 (P82534)	ITCGQVSSSLAPCI ¹ GVVSGGAVPFA-C ² CGN ³ IRNVN ⁴ LART ⁵ ADRAACNCLKQLSGSIPGVNPNNAAAL ⁷ PKCGVNPV ⁸ Y--KISASTNCATVK	90
Pru av 3 (Q9M5X8)	LTCGQVSSSLAPCI ¹ GVVRGGGAVPFA-C ² CGN ³ IRNVN ⁴ LART ⁵ ADRTACNCLKQLSASVPGVNPNNAAAL ⁷ PKCGVNPV ⁸ Y--KISASTNCATVK	88
Fra a 3 (AAV83346)	ITCGQVTHNVAPCFN ¹ VKSGGAVPFA-C ² CKGV ³ SLNSMAKTTADRTQTCNCLKSAAGSIKGLNANLAAGL ⁷ PKCGVNPV ⁸ Y--KISTSTNCNNVK	63
Vit v 1 (AAO33394)	VTGQVASALSPT ¹ SVLQKGGAVPAG-C ² CSGIKSLNSAAKTTGDRQAACKCLKTFSSSVSGINYLGLASGL ⁷ PKCGVNPV ⁸ Y--KISPSTDCSKVT	62
Cit s 3 (Q6EV47)	ITCGQVTGSLAPCI ¹ VLLSGGPIVFP-C ² CGNV ³ SLNSAAKTTTPDR ⁶ TACNCLKQAAGSIPNLNPNNAVGLPRA ⁷ CGVSI ⁸ PY--KISISTDCSKVR	67
Lyc e 3 (CAJ19705)	LTCGQVSTSLAPCI ¹ LYLMNRPL-RN-CCDGVKGLLQAKTTVDRAACTCLKSAASFTGLNLGKAAALPNT ⁷ CSN ⁸ LYF--KISPSTDCSKVQ	53
Cor a 8 (AAK28533)	LTCPIKIGNLT ¹ PCVLLKNGVLPES-C ² CKGV ³ RAVNDASRTTSDRGSACNCLKDTAKGIAGLNPNLAAGL ⁷ PKCGVNPV ⁸ Y--KISPSTNCNNVK	59
Zea m 14 (P19656)	AISCGQVASAIAPCI ¹ SVARGQSGPSAGCCSGVSLNNAARTTADRAACNCLKNAAGVSGNLNAGNAASIPSKCGVSI ⁸ PY--TISTSTDCSRVN	63
Hev b 12 (AAL25839)	ITCGQVQSALVPCLS ¹ YLKTTPTPTATCCNGVETINNAKTTADERTACQCLKSAAGSVKGLNPPTVAPGL ⁷ SKCGVNPV ⁸ Y--KISLSTNCATVK	65
Par j 1 (O04404)	QETCGTVVRALMPCLE ¹ FTQKEKEPSKGCSSGAKRLDGETKGLQ ² VHACECIQTAMKTYSDIDGKLVEVPKHC ³ IGVDSKLPPI ⁴ DVNMDCKTLGV	29
Par j 2 (O04403)	BGPGKVHHIMPCLE ¹ FTKEEKEPSKSCSGTKKLSEVKT ² BQKEACKCI ³ VAAATKGISGKNELVAEVPK ⁴ CGITTT-LPPI ⁵ TADFDCSKIES	28
Amb a 6 (U89793)	PTCDTVQNILAPCAGFLT ¹ QEQ ² -ESKACCTGVN ³ ILNLSRKT ⁴ KAE ⁵ AVAVNCNIKELTKSIA-YDPKRMPLSTK ⁶ CG-VK ⁷ EDFPAVDKNLDCSKLPV	34

Fig. 2. Alignment of amino acid sequences of nsLTP allergens. Only those members of Table 1 whose full sequence is available are included in the figure. Accession numbers are indicated in parenthesis. Regions corresponding to the IgE-epitopes located in peach Pru p 3 are marked in green, and percentages of sequence identity (% id) with respect to this allergen are shown. The C-terminal domain of Par j 1 and Par j 2 allergens is not included.

and plane tree Pla a 3 are clearly implicated in plant food-pollen co-sensitization, displaying partial cross-reactivity with peach Pru p 3 and other food allergens [96,109–111,119,120]. The former shares 40% of its determined N-terminal sequence with the peach allergen and has a prevalence of specific IgE around 70% in Artemisia-allergic patients [109,110]. Sensitization to Pla a 3 is low in plane tree pollen-allergic subjects without food allergy, but high (>60%) among those with associated peach allergy [111]. A second type of allergenic nsLTP from pollens corresponds to ragweed Amb a 6 and olive Ole e 7, which are minor allergens. These present less than 35% of sequence identity with Pru p 3, and have not been involved in pollen-plant food cross reactivity [106–108]. Interestingly, Ole e 7 reaches higher prevalence values (up to 50%) in areas with strong counts of olive pollen, where it is also significantly linked to severe clinical symptoms (D. Barber et al., unpublished). Finally, the major allergens of *Parietaria*, Par j 1 and Par j 2, represent a further type of nsLTP members. Both show larger molecular sizes of typical 9 kDa nsLTP allergens—14.7 kDa (139 amino acid residues) and 11.3 kDa (102 residues) for Par j 1 and Par j 2, respectively—although conserving the 4 disulphide bridges of the nsLTP fold, low levels of sequence identity (26–29%) with peach Pru p 3, and no reported cross-reaction with any food or even pollen (Art v 3) allergens of the family [102–105,110].

3.2. Recombinant allergens, diagnostic tools and strategies for hypoallergenicity

The isolation of cDNA clones coding nsLTP allergens (see Fig. 2) and the production of recombinant forms in heterologous expression systems have enabled not only their further structural characterization (deducing full amino acid sequences and achieving 3-D structural modelling), but also their use in allergen component-based *in vitro* diagnosis [121]. Up to 10 nsLTP recombinant allergens from different food and pollen sources have been obtained, using prokaryotic (*Escherichia coli* for Par j 1, Pru a v 3, Hev b 12, Cor a 8 and Lyc e 3) [89,93,101,122,123] or eukaryotic (*Pichia pastoris* for Am b 6, Pru p 3, Mal d 3, Cit s 3, and Fra a 3) [90,92,107,124,125] heterologous expression systems. An increasing number of studies are using some of these recombinant forms as diagnostic tools for plant food- and/

or pollen-allergic patients [96,123,126,127], although an extensive validation of the immunological equivalence with their corresponding natural counterparts has rarely been performed. An example is the comparison of the global folding, IgE binding potency and biological capacity of the natural and recombinant versions of peach Pru p 3, that has allowed to confirm such equivalence [128].

The availability of cDNA clones and recombinant allergens has also opened the way to generate hypoallergenic nsLTP mutants, for potential use in specific immunotherapy. The aim of this approach is to lower the IgE reactivity without affecting the T-cell response, thus reducing the risk of severe IgE-mediated side-effects and directing the repertoire of T-cell specificities [129]. Site-directed mutagenesis of the critical amino acid residues responsible for IgE-binding, but not for T-cell activation, would eventually lead to potential hypoallergenic variants. Obviously, a previous knowledge of T-cell epitopes, and especially sequential and conformational B (IgE) epitopes, is required to design the engineered allergen. This information is very limited for IgE and non-existing for T-cell epitopes in the case of the nsLTP panallergen family. Three sequential IgE epitopes, covering residues 11–25, 31–45, and 71–80 (see Figs. 1 and 2), have been mapped in peach Pru p 3 [130], and similar regions (residues 2–23, 27–46 and 62–84) located in latex Hev b 12 [131]. In addition, the 3-D modelling of the peach allergen has allowed to identify the positions R39, T40 and R44 at the end of the second epitope as putative critical residues and, therefore, the triple mutant R39A/T40A/R44A was produced. This potential hypoallergenic variant shows a 5-fold decrease in IgE-binding potency as compared with recombinant Pru p 3 [130], but, unfortunately, the decrease does not seem to be reflected in a lower biological activity as determined by means of basophil activation and histamine release tests (G. Salcedo et al, unpublished).

In this context, the distant from Pru p 3 and non-crossreactive allergen Par j 1 of *Parietaria judaica* pollen has also been the target of several studies. Its 3-D structural model has led to locate a conformational and immunodominant IgE epitope in its N-terminal region (residues 1–30), which includes critical residues: two cysteines (C14 and C29) forming a disulphide bridge and several charged amino acids (K21, K23, E24 and K27). Site-directed mutagenesis of these residues

demonstrated their importance in IgE-binding, as well as the role of the cysteine pairing in the maintenance of the dominant IgE epitope [132]. Par j 1 hypoallergenic variants have been generated by disruption (Cys→Ser mutation) of the disulphide bridges, and those lacking 3 or 4 bridges display low, or even no, IgE-binding capacity [133] and show reduced allergenicity. However, they retained IgG and T-cell recognition when tested in a murine model [134]. Additional IgE epitopes have later been detected in Par j 1 (residues 18–37, 41–47, 72–79 and 118–125), some of them being highly similar to those found in the homologous allergen Par j 2 [135].

Alternative strategies to achieve hypoallergenicity of consumed fruits containing nsLTPs have recently been explored. One approach is based on genetic engineering techniques to decrease the amount of allergen in transgenic food, whereas others try to take advantage of the natural variability in allergen levels among fruits from different cultivars, storage conditions, etc. or of the accumulation of nsLTPs in fruit peels. Silencing of tomato *Lyc e 3* genes using the double-stranded RNA interference method has allowed to obtain transgenic tomatoes with a drastically reduced *Lyc e 3* level (<0.5% of that found in non-transformed fruits) [93]. Preliminary data indicate that a significant reduction of allergenic potency, determined both *in vitro* and *in vivo* (skin prick tests), has been achieved in the transgenic fruit [93,136]. On the other hand, the development of different ELISA methods using monoclonal and/or polyclonal antibodies against peach Pru p 3 and apple Mal d 3 [125,137,138] has greatly helped to quantify the allergen load in these *Rosaceae* fruits. A screening of 15 peach and nectarine cultivars (O. Ahrazem et al, unpublished) has confirmed the accumulation of Pru p 3 in peels, showing a mean level around 220-fold higher than pulps (132.8 versus 0.6 µg/g of fresh weight). Therefore, industrial peeling of fruits can be a potential treatment to drastically reduce their allergenicity, as has been proposed [139]. Furthermore, there are significant differences in whole fruit allergen content (from 5.5 to 41.1 µg/g of fresh weight) among cultivars from different origins, thus suggesting a genetic component in the variability recorded. In fact, different expression patterns of Pru p 3 in the epicarp of ripe fruits has been detected [140]. Intercultivar differences in the amount of apple Mal d 3 has also been reported [125,141]. Moreover, physiological (both *Rosaceae* fruit allergens showed a progressive increase during ripening) and environmental (e.g. storage conditions) factors affect the allergen levels in both *Rosaceae* fruits [139,141]. Thus, cultivar selection and optimization of harvesting and post-harvest handling could contribute to reduce allergen loads in some plant foods.

3.3. nsLTPs as a true food allergen model

Several characteristics of the nsLTP panallergen family have led to propose it as a model of true food allergens [8,11]. Its resistance to proteolytic digestion (and heat treatment) suggests that its allergenic members retain their immunogenic and allergenic motifs after passing through the gastrointestinal tract, and can interact with the associated epithelial immune system to induce both sensitization and systemic symptoms.

3.3.1. Resistance to protease digestion and heat treatments

Resistance to simulated gastric fluid (pepsin at acidic pH) digestion has been demonstrated in several allergenic nsLTPs, such as peach Pru p 3, cherry Pru av 3, grape Vit v 1 and barley nsLTP1 [114,137,142–144]. The proteins remain intact after up to 2 h of treatment and even after 24 h around 40% of the allergen (in the case of Pru p 3) has not been processed [137]. Recently, an *in vitro* model of gastrointestinal digestion to test the proteolytic stability of Vit v 1 has confirmed this resistance, and shown that duodenal digestion (bile salt plus trypsin and chymotrypsin) trims off a C-terminal fragment, producing a 6-kDa peptide which maintains the IgE-binding capacity [144]. Both simulated gastric and duodenal treated samples have a similar biological activity (basophil histamine release potency) and *in vitro* response as the native allergens [143,144].

nsLTPs tested so far also resist heat treatments with temperatures up to 100 °C [142,143,145–148]. Peach Pru p 3 and maize Zea m 14 still bind IgE after 30 min at 121 °C and 2 h at 100 °C, respectively [145,146], but more accurate determinations have revealed a significant decrease (around 30-fold) in the IgE-binding and biological activity of apple Mal d 3 after 2 h at 100 °C [148]. However, boiled (100 °C for 30 min) and baked (180 °C for 20 min) apples and apple peels containing this allergen trigger *in vivo* responses (skin prick tests and oral challenges) in fruit nsLTP-sensitized patients [147].

Interestingly, the stability to heat treatment and proteolytic resistance explains the presence of active allergen forms of nsLTPs in processed plant products: beverages, such as beer [118] and wine [91,149], peach- and cherry-derived products (juice, jam) [137,143,145], and even eating heat-treated maize foodstuffs [100,146].

3.3.2. Association with severe systemic clinical symptoms

The high stability of the above-mentioned immunogenic conformation seems to be the most important factor contributing to the association between nsLTP sensitization and severe systemic reactions (generalized urticaria, angioedema, asthma, anaphylaxis, etc.) [8–11]. Such association has mainly been observed in patients from the Mediterranean area (Spain and Italy) with allergy to several plant foods, such as peach [82], apple [83], cherry [127], hazelnut [97] and maize [100]. Thus, it has been determined that sensitization to Mal d 3 and Pru av 3 is a risk factor for systemic reactions in Spanish subjects with IgE-mediated reactions to apple and cherry, respectively [83,127]. In contrast, plant food allergens linked to birch pollen-related food allergy, mostly members of the PR-10 and profilin families, are commonly associated with mild and local symptoms (oral allergy syndrome) [74,75,83,127]. This is likely due to the fact that they are labile allergens, readily hydrolyzed by digestive proteases [78,143,150].

3.3.3. Different populational profiles of sensitization to nsLTPs across Europe

Unexpected and drastic differences in the prevalence of sensitization to nsLTPs have been found between the populations of North and Central Europe and that of the Mediterranean area. Some examples of these differential sensitization patterns

Table 2

Sensitization patterns to plant food allergens belonging to the nsLTP, PR-10 and profilin family in patients with allergy to Rosaceae fruits from different European geographic areas

Offending fruit	Number of patients	Country	Sensitization (%) to			Reference
			LTPs	PR-10	Profilin	
Peach	76	Spain	62	7	34	[82]
Cherry	121	Switzerland and Germany	11	96	18	[127]
Apple	22	Spain	91	5	9	[83]
	99	Netherlands	1	70	13	
	94	Austria	2	84	11	
	99	Spain	47	16	41	

are shown in Table 2. The nsLTP allergen family is particularly important in south European countries (data currently available from Spain and Italy), whereas its prevalence in Central Europe is very limited (<15%). This profile has been detected in patients allergic to peach, apple, cherry, hazelnut, and even to *Parietaria* pollen [82,83,97,127,151]. Moreover, cherry-allergic patients from Germany and Switzerland sensitized to Pru av 3 (cherry nsLTP) seem to be clinically different from Spanish subjects with sensitization to this allergen, since no clear correlation with the severity of symptoms has been found in the former [127].

nsLTP allergens from several foods, including peach [82], cherry [127], asparagus [94], lettuce [95], cabbage [96], hazelnut [97], walnut [98], chestnut [99] and maize [100], as well as pollens, such as *Artemisia vulgaris* [110] and *Parietaria judaica* [151], have a prevalence above 50% in Mediterranean patients showing IgE-mediated reactions to the corresponding plant source. An array of sensitization profiles to nsLTP allergens have been described in these patients, although the reactions are frequently associated with allergy to peach Pru p 3, which has been suggested to be the most likely sensitizing agent. Thus, among patients reporting peach allergy, few are monosensitized to Pru p 3, some are only sensitized to Rosaceae fruit nsLTPs, and others react also with homologous allergens from different plant foods and pollens (i.e. *Artemisia* and *Platanus*) [83,96,99,111,115,119]. It should be noted that members of the nsLTP family from orange [92], strawberry [90] and tomato [93] present prevalence values below 50% in the corresponding groups of allergic subjects tested so far, thus indicating that nsLTPs can be minor allergens in some plant foods, even in the Mediterranean population.

The drastic differences in sensitization to nsLTP allergens between Central European and Mediterranean populations are yet to be fully clarified. Genetic factors (not described so far), differences in dietary habits (time of Rosaceae fruit introduction in the diet, amount and year/period of consumption, etc.), and level and composition of pollen exposure have been claimed to account for such differences. A higher consumption rate of peaches, both as fresh fruit and ready-made meals, by Spanish children as compared with those from North and Central Europe has been suggested [83]. On the other hand, data now available indicate that nsLTP sensitization is mainly located in areas free

of birch (Fagales) pollen, where PR-10 proteins are the most relevant allergens [12,13,77,78]. In contrast, a potential association between sensitization to food nsLTPs and specific IgE to mugwort and plane tree pollens have been uncovered [95,96,109–111]. Thus, inhalation has been proposed as a second route of sensitization, based on the clear cross-reactivity between peach Pru p 3 and its homologous allergens from these pollens (mugwort Art v 3 and plane tree Pla a 3) and on evidence supporting the fact that Art v 3 can be the primary sensitizing agent in some patients with mugwort and peach allergy [110]. Moreover, Pru p 3 inhalation from peach leaves could occur prior to the clinical manifestation provoked by this allergen via the oral route [152]. Future research at different levels (nutritional, clinical, biochemical, etc.) will help to clarify the different routes and geographic patterns of sensitization to nsLTP allergens.

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