

Research paper

The lipid transfer proteins (LTP) essentially concentrate in the skin of Rosaceae fruits as cell surface exposed allergens

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Abstract

The localization and distribution of non-specific lipid transfer proteins (nsLTP) allergens in the skin and pulp of Rosaceae fruits (apple, peach, apricot, plum) has been investigated. nsLTP essentially concentrate in the pericarp of the fruits whereas the pulp contains lower amounts of allergens. Immunolocalization showed they are primarily located in the cytosol but are subsequently excreted and finally accumulate at the plasmalemma–cell wall interface and in the cell wall. However, high discrepancies were observed in the content of allergens among, e.g. different cultivars of apple. As a consequence, the consumption of peeled-off fruits is recommended to reduce the risk of severe allergic reactions (anaphylactic shock) in individuals sensitized to Rosaceae fruits.

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Keywords: Mal d 3; Pru p 3; Pru d 3; Pru ar 3; Allergen; Lipid transfer protein; Rosaceae fruit

1. Introduction

Non-specific lipid transfer proteins (nsLTP) have been recently introduced as major allergens in food allergy, especially in allergies to the Rosaceae (apple, apricot, peach, plum) fruits [1–8]. Plant nsLTP consist of small-sized basic proteins ($pI \geq 9$) of either 91 (LTP2 group) or fewer (LTP1 group) amino acid residues that belong to family 14 of pathogenesis-related (PR-14) proteins [9]. nsLTP allergens, which essentially occur in LTP2 group, consist of a core made of four α -helices stabilized by four conserved disulfide bridges and a loosely coiled C-terminal tail [10–18]. This tightly packed three-dimensional structure accounts for the

extreme resistance of nsLTP to both thermal denaturation and proteolysis [19–22]. Accordingly, nsLTP from food products trigger hypersensitive reactions, e.g. contact dermatitis, in individuals previously sensitized by pollen nsLTP but are also capable to achieve the sensitization of susceptible individuals via a stimulation of the lymph nodes of the gastro-intestinal tract immune system [23,24]. The eventual occurrence of allergens at the cell surface is of particular importance to promote contact dermatitis reactions in individuals sensitized to fruit allergens [25]. Previous measurements have pointed out some discrepancies among the amount of nsLTP in the skin and pulp of apple fruit depending on the apple varieties [26]. In most of the varieties, the skin of apple fruits exhibited the higher content in nsLTP. In the present work, we completed these previous informations by focusing our attention on the content of LTP in different Rosaceae fruits (apple, peach, apricot, plum) and, especially, on the localization of nsLTP at the cell surface. With the exception of the plum fruits, the skin of other Rosaceae fruits usually contains the higher amounts of nsLTP and, more interestingly, most of the allergen occurs at the cell surface and is therefore directly available to trigger contact dermatitis in sensitized people. Such a particular localization accounts for the unexpected quick allergenic response of sub-

Abbreviations: Ab, antibodies; ELISA, enzyme linked immuno-sorbent assay; LTP, lipid transfer protein; nsLTP, non-specific lipid transfer protein; Mal d 3, *Malus domestica* (apple) allergen 3; PBSTM, 10 mM phosphate buffered saline (pH 7.4) containing 0.2% (v/v) Tween 20 and 5% (v/v) defatted milk; Pru ar 3, *Prunus armeniaca* (apricot) allergen 1; Pru d 3, *Prunus domestica* (plum) allergen 1; Pru p 3, *Prunus persica* (peach) allergen 3; PVP, polyvinyl pyrrolidone; TBS, 20 mM Tris buffered saline (pH 7.5).

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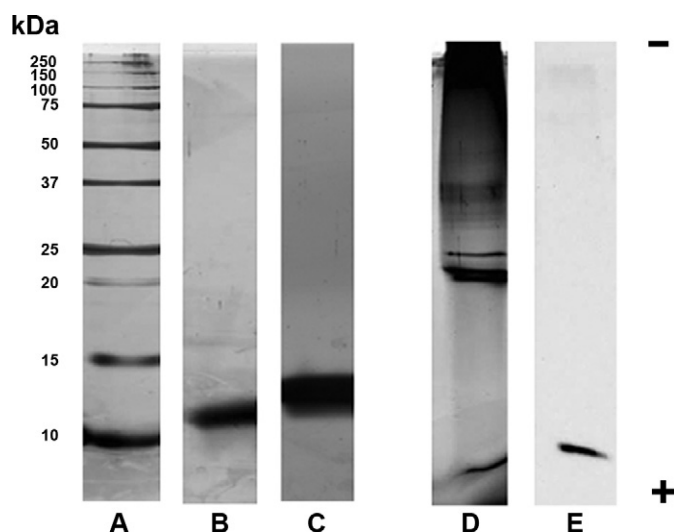


Fig. 1. Silver nitrate-stained SDS-PAGE analysis of purified Pru p 3 (B), Mal d 3 (C), soluble proteins of peach (D), and molecular weight markers (A). Immunoblot analysis of total soluble proteins from peach (E) showing that the rabbit anti-Pru p 3 polyclonal antibodies recognize a single band and does not react with any other protein fraction.

jects previously sensitized to peach upon a simple skin contact with the fruit.

2. Results

As shown from immuno-tissue-prints (ITP) experiments, nsLTP are essentially concentrated in the skin of apple and peach fruits whereas this allergen seems to be similarly distributed in both the skin and pulp of apricot and plum fruits (Fig. 2). Enzyme linked immuno-sorbent assay (ELISA) measurements confirm that the skin of peach and apple contains the higher amounts of nsLTP but discrepancies occur between different fruit varieties (Table 1). Especially in peach, the downy skin of the fruit consists of short hairs extremely rich in LTP as shown from immuno-cytochemical observations (Figs. 3H, K and 4D). In fact, the LTP amount of the skin is underestimated since the fruits were carefully washed before preparing the protein extracts that should eliminate most of the LTP-rich downy cover of the fruits. Accordingly, the LTP content of the skin measured on unwashed peach fruits was definitely higher ($\sim 8.4 \mu\text{g g}^{-1}$) than that measured for washed skin ($\sim 5.2 \mu\text{g g}^{-1}$). The ratio of the nsLTP content in the skin and pulp thus reaches 2.5 instead of 1.6. Very similar results were reported for other peach varieties [25]. The discrepancies observed between different varieties of apple are extremely important. Compared to a few varieties (Chanteclerc, Golden delicious, Granny Smith) where the content of nsLTP of the skin amounts from 10- up to 30-times that measured in the pulp, the ratio of the nsLTP content in the skin and pulp of other apple varieties varies from 2 to 4 (Table 1).

The immuno-cytochemical localization of nsLTP in the different parts (epidermis, hypodermis, parenchyma) of the fruit pericarp has been documented using polyclonal rabbit antibodies (Ab) specifically directed against Pru p 3 and Mal d 3

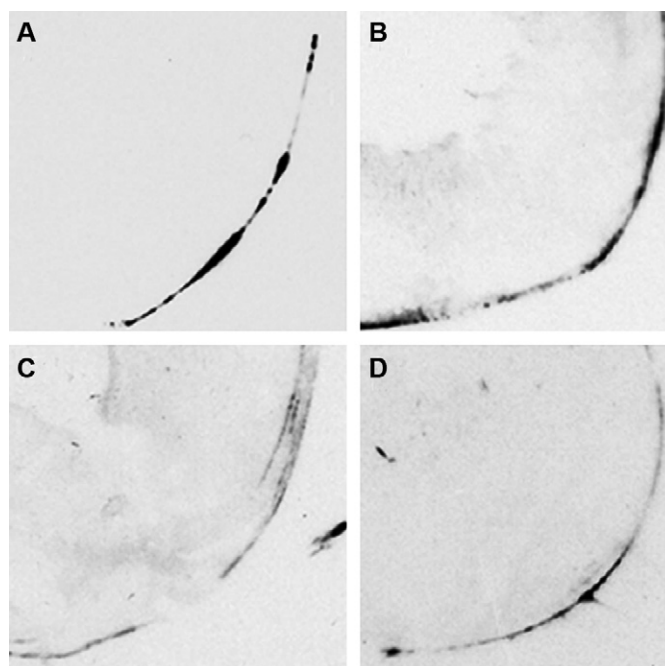


Fig. 2. ITP performed on apple (Golden delicious) (A), peach (B), yellow plum (C) and red plum (D).

Table 1

LTP content (expressed as $\mu\text{g g}^{-1}$ tissue) in the skin and pulp of fruits

Fruit	LTP content of skin ($\mu\text{g g}^{-1}$ tissue)	LTP content of pulp ($\mu\text{g g}^{-1}$ tissue)	Ratio skin/pulp
Royal Gala	3.3	1.2	2.8
Bertanne	2	0.6	3.3
Pink Lady	7.9	1.1	7.1
Granny Smith	17.3	0.5	34.6
Chanteclerc	12.4	1.2	10.3
Golden delicious	12.0	0.9	13.3
Fuji	9	1.1	8.2
Red Chief	3.4	1.8	1.9
Reinette Canada	2	0.5	4
Apricot	1.8	1.6	1.1
Peach*	8.4	3.4	2.5
Peach**	5.2	3.2	1.6
Yellow plum	1.5	1.7	0.9
Red plum	1.5	1.7	0.9

Measurements performed on unwashed (*) and washed (**) peach skin.

(Fig. 1). Confocal laser scanning microscopy observations of apple samples showed that nsLTP labeling essentially occurred in the cytoplasm in contact with the cell wall (Fig. 3G, J). In addition, the labeling strongly decreased from the pericarp and, especially, from the hypodermal cells to the parenchyma cells of the mesocarp. A rather heterogeneous and irregular labeling was observed in peach and plum samples, with spots of fluorescence distributed within the cytoplasm and the inner part of the cell wall (Fig. 3H–L). Especially in peach, a very strong labeling was associated to the thick wall of the downy hairs covering the epidermal cell layer (Fig. 3H, K).

A more detailed subcellular localization of nsLTP in fruit was carried out by electron microscopy using immuno-gold labeling. Although some discrepancies occurred among the dif-

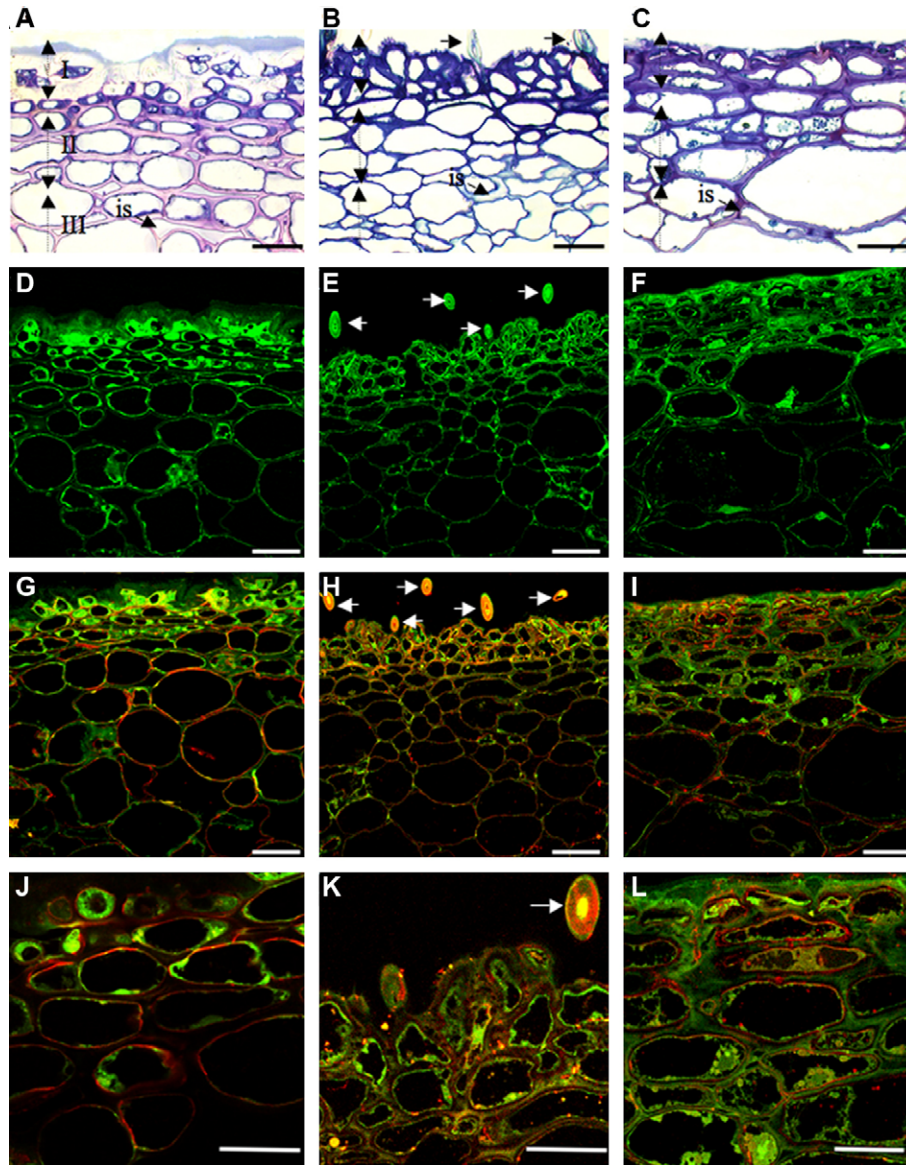


Fig. 3. A–C: Bright field microscopy of semi-thin sections stained with toluidine blue showing the cell layers forming the epidermis (I), hypodermis (II) and parenchyma (III) of the pericarp of apple (A), peach (B) and plum (C). Intercellular spaces (is) are indicated. The arrows in B indicate the hairy epidermal cells of peach fruit.

D–L: Confocal laser scanning microscopy showing the immunofluorescence labeling (red) of nsLTP in apple (D, G, J), peach (E, H, K) and plum (F, I, L). All images correspond to the maximal projection of 10–15 confocal planes. In control samples (D, E and F) lacking the primary antibody, only the green autofluorescence is visible. Arrows in E, H and K indicate sections of hairy epidermal cells of peach. Scale bar = 50 μm .

ferent fruits, gold particles were mainly associated to the inner part of the cell wall in contact with the plasmalemma and to the cell wall itself in the different cell layers of the pericarp. In apple, only a few gold particles were detected within the thick cell wall of epidermal cells suggesting a rather low accumulation of nsLTP in this cell layer (Fig. 4A). A much more dense labeling occurred in the cytoplasm and the inner part of the cell wall of hypodermal cells (Fig. 4B). Gold particles were similarly distributed within the cytoplasm and inner part of the cell wall of parenchyma cells (Fig. 4C) while the outer part of the cell wall in contact with intercellular spaces exhibited a faint labeling (Fig. 4C). A rather different labeling was

observed in peach pericarp samples. In full agreement with previous confocal microscopy observations, the most striking feature concerns the strong labeling of the hairy cell walls (Fig. 4D) that suggests a high concentration of nsLTP in the downy hairs covering the fruit epidermis. In hypodermal cells, labeling occurred in both the cell wall and the cytoplasm (Fig. 4E) whereas gold particles were mainly located in the cytoplasm in contact with the cell wall, and, more scarcely, within the cell wall of the large parenchyma cells (Fig. 4F). In plum, gold particles appeared as mainly associated to the cell wall of both the epidermal, hypodermal and parenchyma cells (Fig. 4G–I).

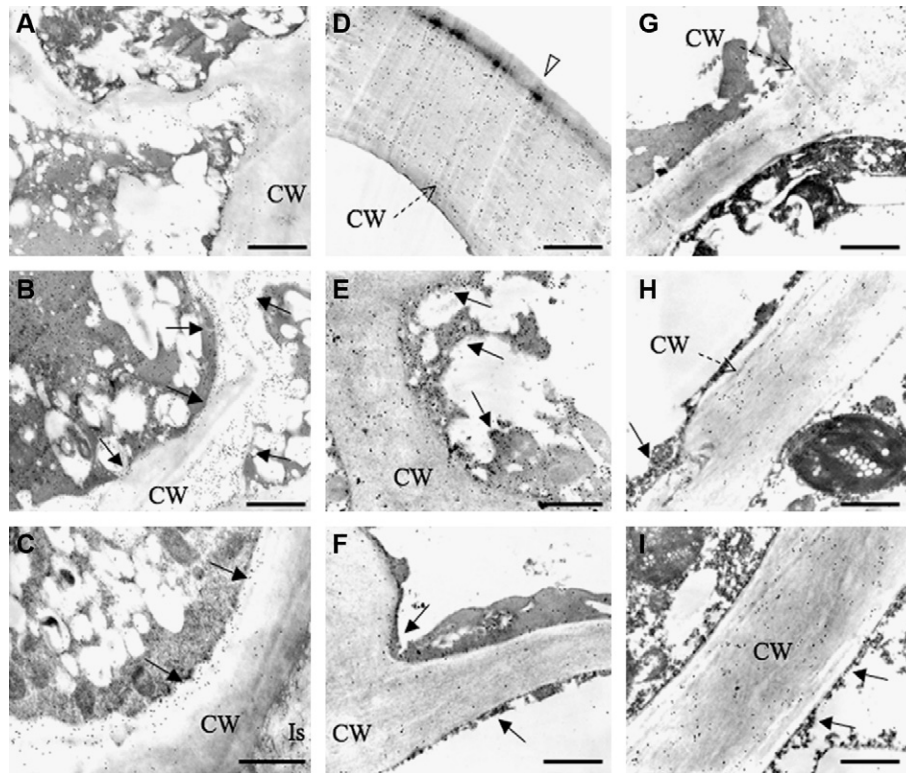


Fig. 4. Ultrastructure and location of nsLTP in the pericarp of apple (A–C), peach (D–F) and plum (G–I) fruits. Localization of nsLTP was detected by immuno-gold labeling in epidermal (A, D, G), hypodermal (B, E, H) and parenchyma (C, F, I) cells of fruit pericarp. Note the dense labeling (black arrows) revealed by gold particles (10 nm) of the inner part of the cell wall (CW) in contact to the plasmalemma (apple) and of the thick wall of hairy epidermal cells of peach. Is, Intercellular space. Scale bar = 1 μ m.

3. Discussion

Except for plum and apricot, other Rosaceae fruits and especially apple, concentrate the nsLTP in the skin. However, discrepancies were observed among the different commercially available apple varieties. In this respect, a few apple varieties like Granny Smith, Golden delicious or Chanteclerc, definitely differ from other by an extremely high content of nsLTP in the skin, which can amount from 10 up to 30-times (Granny Smith) that measured in the pulp (Table 1). In other apple varieties, a moderate ratio between the nsLTP content of the skin and the pulp remains in the range of 2 to 4. Similar discrepancies were observed among the fruits from different peach [25] and apple [26] varieties.

Such a cell wall localization of nsLTP observed in Rosaceae fruits suggests that once synthesized, the strongly basic nsLTP (calculated pI of 9.14 for Mal d 3 and Pru p 3, 9.33 for Pru d 3, and 9.36 for Pru ar 3) excreted from the cytosol at the wall-plasmalemma interface should be further entrapped within the acidic pectin matrix of the cell wall to behave as surface exposed allergens. Mapping of electrostatic potentials on the molecular surface of the modeled nsLTP from fruits revealed a predominantly electropositively charged surface susceptible to readily interact with electronegative counter-parts (Fig. 5). A similar excretion of a nsLTP previously synthesized in cytosol and further localized in the cell wall was reported for other nsLTP [27] and, especially, for Par j 1, another major nsLTP

allergen of the pollen grains from the Pellitory-of-the-wall *Parietaria judaica* [28]. Obviously, the small size of nsLTP should favor their migration to the cell wall once they have been excreted from the cells. In addition, their reported resistance to proteolytic enzymes [19,22] should prevent their degradation and preserve their allergenic potency.

The subcellular localization of nsLTP in Rosaceae fruits shed new lights on the availability of these allergens for triggering an anaphylactic response in susceptible individuals. The localization of secreted nsLTP as components of the cell wall of epidermal/hypodermal cells ensures the exposition of these allergens at the surface of the Rosaceae fruits. Accordingly, they are susceptible to directly interact with the skin or the buccal mucosae of the sensitized individuals to trigger a quick hypersensitive response. In this respect, anaphylactic responses such as contact urticaria and oral allergy syndrome have been observed in sensitized Spanish patients just after a skin contact with peach fruits in relation, most probably, with the high nsLTP content of the downy hairs covering the fruits [29]. Recent results suggest that the fuzzy hairy cover of peach might account for the high prevalence of sensitization to peach in people from Mediterranean countries [30]. As a practical consequence, both the handling and consumption of previously peeled-off peach or apple fruits should be recommended to weakly sensitized people to significantly reduce the risk of a severe allergic disease.

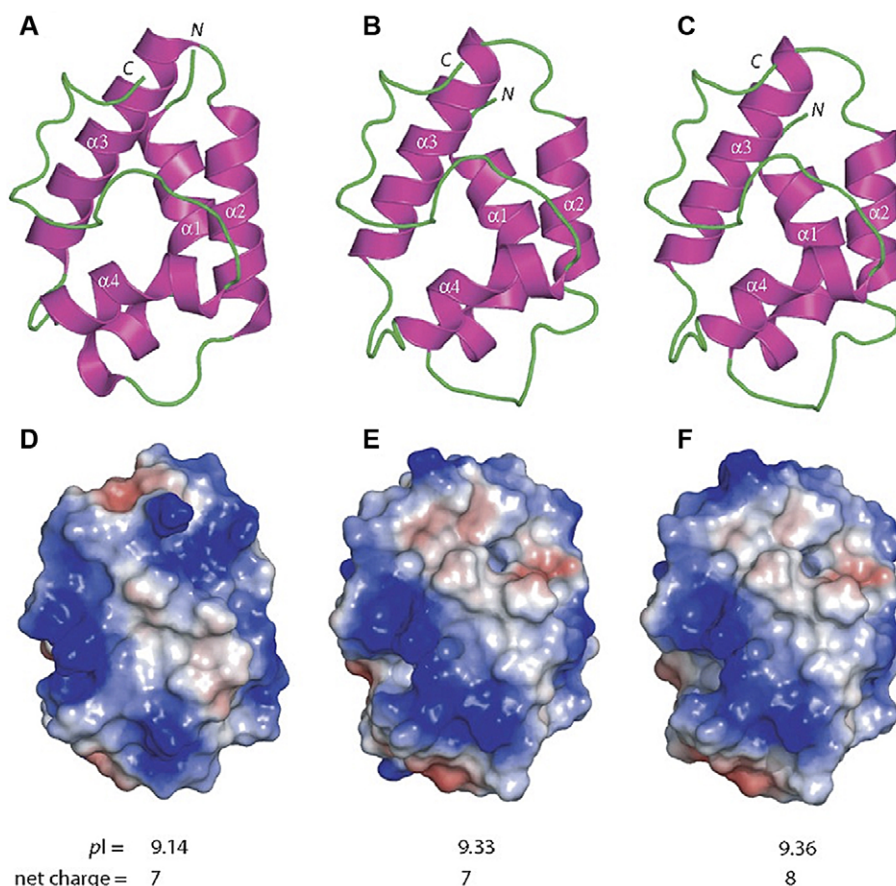


Fig. 5. Ribbon diagrams of the three-dimensional structure of Pru p 3 (PBD code 2ALG) [18] (A) and three-dimensional models built for Mal d 3 (B) and Pru ar 3 (C). Mapping of electrostatic potentials on the molecular surface of Pru p 3 (D), Mal d 3 (E) and Pru ar 3 (F). N and C indicate the N- and C-terminus of the polypeptide chains, respectively. Alpha-helices are numbered $\alpha 1$, $\alpha 2$, $\alpha 3$ and $\alpha 4$ according to their occurrence from the N-terminus of the polypeptide chains. Calculated values for the pI and net charge are indicated under the electrostatic potential maps.

The occurrence of nsLTP as a surface exposed protein has been also reported for other vegetative organs of plants. In this respect, nsLTP consists of the major protein of the waxy covering of the broccoli (*Brassica oleracea*) leaves [31,32].

In the present work, the ELISA measurements and immunocytochemical localization of nsLTP were performed on ripe fruits of different commercially available fruit varieties but the nsLTP content of peach was recently reported to highly depend on the degree of fruit ripening [33]. Moreover, a few preliminary results obtained on apple varieties issued from biological agriculture suggested some interesting discrepancies that deserve more deeply investigations. This task is now in progress in our laboratory.

4. Materials and methods

4.1. Isolation of LTP

nsLTP were purified from the skin and pulp of apple, peach, apricot and plum fruits. Usually, 200 g of skin/pulp previously frozen in liquid nitrogen were finely ground in mortar and pestle and suspended in 1 l of 20 mM Tris buffered saline (TBS) containing 2 mM EDTA and 3% (w/v) insoluble polyvinyl pyr-

rolidone (PVP) (Polyclar AT, Sigma) (pH 7.5). The slurry was stirred overnight at 4 °C and then centrifuged 20 min at 12,000 $\times g$ and 20 min at 27,000 $\times g$. The clear supernatant was poured in SpectraPor dialysis tubing (molecular weight cut-off 3500 Da) and dialyzed against dist. wt for 72 h at 4 °C. After centrifugation at 27,000 $\times g$ for 20 min at 4 °C, the supernatant was lyophilized and stored at -80 °C until used.

4.2. Analytical methods

nsLTP were separated by SDS-PAGE in 15% polyacrylamide gels using Tris-aurine as trailing ion [34] and stained with silver nitrate [35] (Fig. 1). Coomassie blue stained bands were digested with trypsin in the gel and mass mapped by MALDI-TOF analysis as previously described [36]. The software Protein Prospector was used for the identification of the protein using the NCBI non-redundant database.

For MALDI-TOF-MS analysis, nsLTP samples of 0.75 μ l of a 0.5 mg ml⁻¹ protein solution in 50 mM Tris buffer (pH 7.5) were co-crystallized on the MALDI plate with 0.75 μ l of 0.6 mM of 3,5-dimethoxy-4-hydroxy cinnamic acid (sinapinic acid) in 50% (w/v) of aceto nitrile containing 0.1% (w/v) trifluoroacetic acid used as a matrix solution. Desorption and

ionization of crystallized samples were carried out on a Voyager-DE™ STR (Perspective Biosystems, USA) mass spectrometer in positive linear mode using an accelerating voltage of 25 kV, a grid voltage of 90% and an extraction delay time of 300 ns. Acquisition mass was performed between 3000 and 25,000 Da using a mixture of three proteins of known mass molecular weight (insulin: 5734 Da, thioredoxin: 11,674 Da and apomyoglobin: 16,952 Da) for the internal calibration.

Far-UV circular dichroism (CD) spectra nsLTP were measured at room temperature on a spectrophotometer Jobin and Yvon CD6 to assess the correct folding of the purified protein samples. The light path for all measurements was 1 mm and concentrations of lectins ranged from 100 up to 300 mg ml⁻¹ in 20 mM phosphate buffer containing 100 mM NaCl (pH 7.4). The proportion of β -strand and α -helical structures was estimated from the CD spectra [37]. According to the absence of β -sheet structures in the nsLTP [10–18] all of the CD spectra showed the occurrence of α -helices and coil structures in fruit nsLTP (result not shown).

The protein content of the nsLTP samples was estimated using the bicinchoninic acid kit reagent (Pierce) [38] with bovine serum albumin (BSA) as a standard.

4.3. Molecular modeling

Molecular modeling of Mal d 3 and Pru ar 3 was carried out on a Silicon Graphics O2 R10000 workstation, using the programs InsightII, Homology and Discover3 (Accelrys, San Diego, CA, USA). The atomic coordinates of the maize LTP Zea m 14 [39] (RCSB Protein Data Bank code 1MZL) were used to build the three-dimensional model of the allergen. The percentages of both identity (~35%) and homology (~75%) Zea m 14 shares with fruit nsLTP allowed us to build rather accurate three-dimensional model using the X-ray coordinates of the maize LTP as a template. Steric conflicts were corrected during the model building procedure using the rotamer library [40] and the search algorithm implemented in the Homology program [41] to maintain proper side-chain orientation. The geometry of loop regions was corrected using the refine option of TurboFrodo [42]. An energy minimization of the final models was carried out by 50 cycles of steepest descent using Discover3. The program TurboFrodo was run to draw the Ramachandran plot and to perform the superposition of the model with the template protein. PROCHECK [43] was used to assess the geometric quality of the three-dimensional models. Cartoons were drawn with PyMOL (W.L. DeLano (<http://www.pymol.org>)).

Electrostatic potentials were calculated using the parse3 parameters [44]. The solvent probe radius used for molecular surfaces was 1.4 Å and a standard 2.0 Å-Stern layer was used to exclude ions from the molecular surface [45]. The inner and outer dielectric constants applied to the protein and the solvent were, respectively, fixed at 4.0 and 80.0 and the calculations were performed keeping a salt concentration of 0.145 M.

4.4. Immune sera

Polyclonal antibodies against apple and peach LTPs were raised in rabbits in the presence of Freund's complete adjuvant (Calbiochem). The allergens (100 µg) freshly dissolved in saline (400 µl) and emulsified with adjuvant (400 µl) were repeatedly injected subcutaneously (200 µl) at 10-day intervals during 4 months. Every 3 weeks, blood was collected and checked for polyclonal antibodies by ELISA. After blood clotting at room temperature and at 4 °C for 4 and 12 h, respectively, the immune sera were collected by centrifugation, filtered through a 0.2 µm membrane and stored at -80 °C until used.

The specificity of polyclonal antibodies was checked by Western immunoblotting on nitrocellulose membrane (Fig. 1). They only revealed a single protein band corresponding to the nsLTP but not reacted with other protein fractions from the fruit extracts. No binding occurred with the rabbit preimmune sera used as a negative control.

4.5. Immuno-tissue-printing

ITP were performed to roughly localize LTP in apple, peach, apricot and plum fruits. Slices (~2 mm in thickness) of fresh ripe fruits thoroughly washed in deionized water were wrapped in filter paper (BioRad) and gently pressed on a 0.2 µm nitrocellulose membrane (Scheicher and Schuel) previously washed with 100 mM ascorbic acid. The membrane was air-dried at room temperature overnight and protein transfer was checked by quick staining of the membrane with 0.2% (w/v) Ponceau red in 0.3% (w/v) TCA. The membrane is overflowed in 10 mM phosphate buffered saline (pH 7.4) containing 0.1% (v/v) Tween 20 and 5% (v/v) defatted milk (PBSTM) during 2 h. After incubation in the presence of polyclonal rabbit immune serum diluted 1:1000 (v/v) in PBSTM during 2 h in a moist chamber, the membrane was washed three-times with PBSTM and finally incubated at room temperature in PBSTM containing the 1:2000 (v/v) diluted goat anti-rabbit IgG immune serum labeled with HRP during 1 h under constant stirring. After three washes with 10 mM phosphate buffered saline (pH 7.4) containing 0.1% (v/v) Tween 20, the membrane is revealed using the ECL Plus detection kit (Amersham) after 3 min exposure in cassette.

4.6. Immunolocalization

Small pieces of pericarp (1–2 mm³) were fixed with 2.5% glutaraldehyde in 0.05 M cacodylate buffer (pH 7.2) for 48 h at 4 °C, and then washed in cacodylate buffer before dehydrated in an alcohol series. The dehydrated samples were infiltrated with graded mixtures of LR White acrylic resin in ethanol (1:2, 1:1, and 2:1) and finally embedded in pure resin. Polymerization was performed overnight at 70 °C. Semi-thin (2 µm in thickness) or ultra-thin (80 nm) sections were prepared using a Reichert ultraCutE microtome. Semi-thin sections were mounted on glass slides for bright field and laser scanning confocal microscopy. Ultra-thin sections were mounted on gold

grids for transmission electron microscopy. Some sections were stained with toluidine blue for bright field microscopy.

Sections were first blocked for 2 h at room temperature in PBST buffer (0.14 M NaCl, 2.7 mM KCl, 7.8 mM Na₂HPO₄, 1.5 mM KH₂PO₄, pH 7.2 plus 1% de Tween 20) containing 1% of BSA. They were incubated overnight at 4 °C with the rabbit antiserum against LTP diluted 1:50 in PBST buffer plus BSA, and then washed in the same buffer. For immunofluorescence labeling, sections were incubated 2 h at room temperature with goat anti-rabbit IgG coupled to Alexa 633 (Molecular Probes) diluted 1:1000 in PBST plus BSA. Sections were then washed and dried. For transmission electron microscopy, goat anti-rabbit IgG coupled to 10 nm gold particles diluted 1:50 in PBST buffer plus BSA (Amersham, RPN422 Auro-Probe EMGAR G10) were used as secondary antibodies. Sections were washed, stained with an aqueous solution of uranyl acetate (5%, w/v), rinsed in water and dried. Primary antibody was omitted in control samples.

Confocal images were acquired with a SP2 confocal laser scanning system equipped with an upright microscope (Leica, Germany) and a 40× (PL APO, N.A. 1.25) oil immersion objective. The 488 nm ray line of an argon laser was used to depict the general feature of the tissue under observation. The emitted autofluorescence of the tissue was collected in the range between 500 and 540 nm. The distribution of the probe Alexa 633 was observed using the 633 nm ray line of a HeNe laser, the emitted fluorescence being collected in the range 645 and 730 nm. The laser line intensities and the PMT settings were kept constant for the control and the labeled samples. No fluorescence was detected between 640 and 700 nm indicating that both the level of autofluorescence and non-specific adsorption of Alexa 633-labeled secondary antibodies are low in this wavelength range. Pictures were computed by projection of 10–15 plan-confocal images acquired in *z* dimension with 0.5 µm increment between two focal planes. Micrographs were performed using a Hitachi-H600 (Japan) transmission electron microscope operating at 100 kV and taken on Kodak-Electron films (Kodak France).

4.7. ELISA measurements

ELISA measurements were performed on standard 96-wells microtitration plates (Limbro) using rabbit polyclonal antibodies against Mal d 3 or Pru p 3. Briefly, the wells were loaded with 50 µl of peel or pulp extracts in 10 mM phosphate buffered saline (pH 7.4) used at various protein concentrations ranging from 0.4 up to 2.0 µg ml⁻¹. The plates were incubated overnight at 4 °C and washed three-times with PBS (pH 7.5). One hundred microliters of PBS (pH 7.5) containing 0.2% Tween 20 (v/v) and 3% BSA (w/v) (PBSTB) were added to each well and the plates were incubated at room temperature for 2 h. Fifty microliters of 10-fold diluted Ab in PBSTB (pH 7.5) were added and the plates were incubated at room temperature under constant stirring. After three washes with PBSTB, 50 µl of 1:2000 (v/v) diluted sheep anti-rabbit IgG coupled alkaline phosphatase (Dako) were added and, after

incubation at room temperature for 45 min, three washes were performed with PBSTB. One hundred microliters of alkaline phosphatase substrate (Sigma) were added and after incubation for 45 min in the dark, 50 µl of 3 M NaOH were added to stop the color reaction. The 405 nm was recorded on a Titer-tek Multiscan spectrophotometer. Each value is the mean of three separate experiments. Appropriate blank and substrate controls were performed under the same conditions.

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