

Electrospray MS and MALDI imaging show that non-specific lipid-transfer proteins (LTPs) in tomato are present as several isoforms and are concentrated in seeds

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Non-specific lipid-transfer proteins (nsLTPs) are major human allergens in many plant species, albeit their role in plant biochemistry is still undefined. They are found in many plant species, either as one or several isoforms according to the species, and usually they are found to concentrate in the outer part of the fruits. In this work, the characterization of tomato nsLTP isoforms was performed on the three main fractions of Piccadilly tomato fruit (peel, pulp and seeds) by using ultracentrifuge devices with molecular cut-off able to retain proteins with molecular weight typical of plant LTPs. The isolated proteins were further analysed by LC-MS, in order to investigate the occurrence and the localization of tomato LTP isoforms. The chromatographic retention times, the molecular masses, the presence of eight cysteine residues in their tertiary structures and the sequence information obtained by MS, although not complete yet, allowed us to identify four different LTP isoforms, not yet reported in the literature, which were found to be concentrated in the seed fractions. None of the molecular masses of these potential LTPs was already present in the UniProtKB/SwissProt database. MALDI imaging experiments confirmed their presence and main localization in seeds, although the actual data hinted at their presence around seeds, rather than exactly in them. These data hint to a complicated scenario concerning LTP proteins in tomato. Copyright © 2014 John Wiley & Sons, Ltd.

Keywords: LC-MS; MALDI imaging; lipid-transfer proteins; Piccadilly tomato; LTP isoforms

Introduction

Non-specific lipid-transfer proteins (nsLTPs) in plants were first described by Kader in 1975^[1] as molecules able to enhance the *in vitro* transfer of phospholipids between membranes and to bind acyl chains.^[2] When they were also found to be synthesized as pre-proteins, containing signal peptides that direct their secretion into the extracellular matrix,^[3] the idea of their possible involvement in intracellular lipid trafficking was subsequently abandoned. On the basis of their observed *in vitro* capacities and of their localization *in vivo*, other roles were suggested, such as in the transport of monomer of cutin, in the deposition of lipophilic cuticular material^[4,5] and in the plant defence mechanisms.^[6] However, 40 years after their discovery, LTPs have yet to be assigned an *in vivo* well-defined biological role. Nonetheless, LTPs have attracted considerable interest because of their importance both in industrial applications (e.g. as foam-stabilizing agent in beer production^[7]) and mostly in human health, where they have been identified as major human allergens in many plant species, also enhanced by their accentuate enzymatic and heat resistances. Particularly in fruits of the *Rosaceae* family, their strong allergenic properties have been clearly observed.^[8] So far, these proteins have been found in many plants and share in common structural and functional properties. They are all soluble, relative small polypeptides (9 kDa for proteins belonging to LTP1 family, 7 kDa for those ones belonging to LTP2 family), containing from 70 to 95 amino

acid residues and with high isoelectric points (9–10), because their primary structure is abundant in charged lysine residues.^[2] When the primary sequences are compared, usually an identity of 40–50% and a similarity of approximately 70% between nsLTPs of various origins are observed.^[9] These high-conserved sequences also possess eight cysteine residues also conserved in similar positions along the primary structure. These eight cysteines, bound to each other, form four disulphide bridges that help the stabilization of the protein tertiary structure.^[2] This background is complicated by the genetic issues related to LTPs, indicating the presence of several genes coding for LTP proteins in single genomes.^[10,11] This multigenic nature yields in many plants many protein isoforms, which show differential tissue developmental expression patterns and possibly different functions. For this reason, in a given plant, one or several isoforms of nsLTPs expressed in different tissues

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and in different moments of the cell development can be found, as in carrot,^[12] maize,^[13] wheat^[14] and many others.^[15–18] Hence, the analysis of where, when and how the LTPs are mostly present may help to elucidate the functions these proteins fulfil *in vivo*. In tomato, several LTP isoforms have been detected so far,^[19–22] mainly in epidermal cells of leaves and in flowers, and very rarely in roots. Moreover, the same proteins also showed IgE binding properties^[22,23] and caused adverse reactions in sensitized subjects, after the ingestion of both raw and cooked tomato fruits.^[24,25] Nevertheless, a full description of these proteins is still lacking and far to be complete. To the best of our knowledge, not many studies have been carried out in order to further investigate the potential existence of other LTP isoforms and their localization and distribution within tomato fruits.^[23] This is of the utmost importance, considering their impact on food safety and the growing importance at clinical level as tomato allergen, and also the widespread use of tomato in the world as food commodity. In this work, tomatoes of Piccadilly variety, which is commonly spread in Italian markets, were used for the characterization of tomato LTP isoforms. The analyses were carried out on the three main fractions of tomato fruit (peel, pulp and seeds) by using ultracentrifuge devices with molecular cut-off able to retain proteins with molecular weight typical of plant LTPs, followed by the characterization of retained proteins by LC-MS, to investigate the occurrence and the localization of tomato LTP isoforms. Four different isoforms, not yet reported in the literature, were found to be concentrated in the seed fractions. MALDI imaging experiments confirmed their presence and main localization in seeds.

Methods

Protein extraction from tomato tissues

Vine tomatoes (Piccadilly variety) were purchased at a local market and were immediately frozen at -20°C up to the analyses. Protein extraction from tomato pulp was performed in 10 mM phosphate buffer solution, following the protocol already described,^[26] while it was slightly modified for the other tissues, as follows: for the peels, tissues were removed from the frozen berries (with a household knife), after a quick rinse with Milli Q water, and the residual pulp was scraped out by a spatula. A total of 10 g of peels was used for the homogenization in 100 ml of cold acetone, in order to allow the full homogenization of the cellulose tissues; for the seeds, after being removed from the locular cavities of the tomato berries, they were placed into a colander and rinsed with Milli Q water to eliminate any residue of pulp tissue. About 7 g of seeds was reduced to a fine powder by milling in a mortar with manual grinding in liquid nitrogen before being homogenized in cold acetone. Protein concentrations were determined using the Qubit® fluorimeter (Technologies Ltd, UK) according to the instructions by the manufacturer.

Protein fractionation in the range 3–30 kDa

Protein fractionation was achieved by Amicon filter devices with nominal molecular weight limit of both 30 and 3 kDa (Millipore, Billerica, MA, USA). Briefly, 3.5 ml of each tissue protein extract was loaded onto a 30 kDa device and centrifuged at 6500 RCF, 4°C for 30 min. The analyses were performed in duplicate for each tomato tissue, in order to allow the characterization of proteins both in the native and in the denatured conformation. After two washes of the membranes with Milli Q water, the filtered solutions were recovered, loaded onto 3 kDa devices and centrifuged at 6500

RCF, 4°C for 45 min, until the depletion of the whole recovered solutions. Two washes with Milli Q water of the retentates (the upper part of the devices) were performed using the same centrifugation settings. Finally, the recovered retentates (about 250 μL) were concentrated under N_2 stream or used for the following analyses.

Reduction and alkylation of native proteins

The volume of the recovered retentates to be treated was measured, the same amount of 8 M urea in 25 mM ammonium bicarbonate solution was added and the mixtures were briefly mixed. A volume of 50 mM dithiothreitol (DTT) in 25 mM ammonium bicarbonate solution was added so that the final concentration of DTT in the samples was 5 M. The mixtures were stirred and were incubated at 60°C for 30 min. After cooling down at room temperature, a volume of 50 mM 2-iodoacetamide in 25 mM ammonium bicarbonate solution was added so that the final concentration of iodoacetamide in the samples was 15 mM. The mixtures were stirred again and allowed to incubate at room temperature for 45 min in the dark. In the end, a volume of the DTT solution was added so that the final DTT concentration was about 3.3 mM, in order to neutralize any remaining 2-iodoacetamide. After a brief shake, samples were concentrated under N_2 stream.

UPLC-ESI-MS analysis of fractionated proteins

Dried samples were resuspended in 200 μL of 0.1% formic acid solution in Milli Q water, briefly mixed by autovortex and centrifuged at 10 000 rpm for 15 min at room temperature. The supernatants were transferred to clean vials and used for the analysis. The chromatographic separation of fractionated protein extracts, both treated and not treated, was performed on an RP column (Acquity UPLC BEH, C8, 1.7 μm , 2.1×150 mm; Waters Corp., Milford, MA, USA) in a UPLC-ESI-MS system (Acquity Ultraperformance UPLC coupled to a single quadrupole mass SQD Waters), using a gradient elution. Eluent A was water with 0.1% formic acid and 0.2% acetonitrile, and eluent B was acetonitrile with 0.1% formic acid; 5 μL of each sample was injected each time, at a 0.15 mL/min flow rate; column temperature 35°C ; sample temperature 18°C ; gradient: 7 min isocratic elution with 100% A, 43 min linear gradient from 0% to 50% of B, 2.60 min isocratic elution with 50% A and 50% B, 0.4 min linear gradient to reach 100% B, 5.20 min isocratic elution with 100% B, 0.8 min linear gradient from 100% to 0% of B and 13 min of reconditioning with 100% of eluent A. ESI-MS conditions were the following: positive ion mode, capillary voltage 3 kV, cone voltage 30 V, source temperature 100°C , desolvation temperature 200°C , spraying gas (N_2) 100 L/h, desolvation gas (N_2) 650 L/h, acquisition time 7–58.2 min, full scan acquisition from 100 to 2000 m/z in continuum mode and 1 s of scan time. Chromatograms and spectra were recorded and analysed by Masslynx software v. 4.1 (Waters). As reference molecule, a purified LTP from peach (Diagnostic Prick Test, Alk Abellö, Copenhagen, DK) was analysed under native conditions, as described earlier.

Size exclusion chromatography analysis of the seed fraction

After being filtered onto a membrane filter (0.45 μm), 10 repetitions of 40 μL of the protein fractionated extract from tomato seeds were applied to a column (300 $\times$ 7.80 mm) of BioSep-SEC-s2000 (Phenomenex, Torrance, CA, USA) attached to a Waters 2695 Separations Module (Waters), equilibrated and eluted with 25 mM sodium phosphate buffer pH 7.0, containing 150 mM NaCl at a flow rate of 1 mL/min.

The eluent was monitored for protein by absorbance at 280 and 214 nm by Waters 2487 Dual λ Absorbance Detector (Waters). The column was calibrated with a set of gel filtration molecular weight standards (Bio-Rad, Hercules, CA, USA). SDS-PAGE of the fractions was performed on 12% gel under reducing conditions by means of Criterion XT precast gel in a Criterion Cell, according to the instructions by the manufacturer (Bio-Rad). Reference markers with known molecular weights (SDS-PAGE Molecular Weight Standards, Broad range, Bio-Rad) were run on the same gel. Gel was stained using CBB R-250 (Bio-Rad) and scanned using a GS800 calibrated densitometer and Quantity One software (Bio-Rad). Fraction containing proteins of interest, further checked by UPLC-MS analysis as described earlier, was dried down on a rotary evaporator (Buchi Rotavapor, Switzerland), resuspended in 250 μ l of Milli Q water and desalted by Amicon filter devices with nominal molecular weight limit of 3 kDa, as described in the text, previously. The desalted retentate was concentrated under N_2 flux.

In-solution trypsin digestion for mass spectrometry characterization

Dried sample to be digested was resuspended in 52 μ l of 25 mM ammonium bicarbonate; 2 μ l was used for determining protein concentration using the Qubit® Fluorometer (Life Technologies Ltd, UK) according to the instructions by the manufacturer. Afterwards, the sample underwent denaturation, reduction and alkylation steps, as previously described. After the urea concentration was lowered to 2 M by adding 25 mM ammonium bicarbonate, trypsin was added in 1:25 ratio (1 mg of trypsin for every 25 mg of protein) to the sample, and it was incubated at 37 °C overnight. The sample was then acidified with 3% formic acid to stop the digestion, and 50 μ l of the digested sample was dried under N_2 stream.

LTQ-Orbitrap analysis of digested proteins

Tryptic peptide analysis was performed with a Dionex Ultimate 3000 micro HPLC coupled with the LTQ-Orbitrap Thermo Scientific™ mass spectrometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) equipped with a conventional ESI source. The source parameters were configured as follows: spray voltage 3.5 kV, capillary voltage 49 V and tube lens 75 V. For the chromatography separation, an Acclaim® PepMap300 C18 (5 μ m, 300 Å, 300 μ m $\times$ 15 cm, Thermo Fisher Scientific Inc.) column was used, and the column oven temperature was set to 25 °C; the separation was run for 82 min using a gradient of 99.8/0.2 H₂O/HCOOH (eluent A) and 99.8/0.2 ACN/HCOOH (eluent B) and a flow rate of 5 μ l/min. The gradient was run as follows: 0–4 min 95% A and 5% B, then to 50% A at 60 min, and 10% A and 90% B at 62, 62–72 min 10% A, followed by the re-equilibration of the column. For MS1 scans, the Orbitrap resolution was 60,000 and the ion population 5×10^5 , with an m/z range from 200 to 1800. For MS/MS in the LTQ, the population ion was 3×10^4 (isolation width of 3 m/z unit). A maximum of four precursor ions (most intense) were selected for activation and subsequent MS/MS analysis. Collisionally induced dissociation (CID) was performed at 35% of the normalized collision energy in all cases (measures by CIM Parma, Italy) and collected by Xcalibur® software (Thermo Fisher Scientific Inc.).

Sample preparation for MALDI-IMS analysis

One fresh Piccadilly variety tomato was included into an aqueous gel of 5% 2-carboxymethylcellulose (CMC) and then left at –80 °C

overnight. Sample inclusion in CMC is helpful to maintain almost intact the internal substructures of the vegetable, allowing freezing it without water swelling and breaks. The following day, the sample was released from CMC embedding, and it was cryo-sectioned at 50 μ m slice thickness using a cryo-microtome (Leica 1850, Leica Microsystems, Nussloch, Germany); the sections were mounted on indium tin oxide conductive glasses (Bruker Daltonics GmbH, Bremen, Germany) and then dried for 3 h to remove the large amount of water present inside the vegetable. A uniform matrix coating was subsequently applied to the dried sample sections using an automatic spraying device (ImagePrep®, software version 2.0, Bruker Daltonics). In order to increase analyte extraction from tissues and uniformity of coating, we decided to use an ionic liquid matrix mixture,^[27–29] composed of 10 g/l α -hydroxy-cinnamic acid and aniline in equimolar quantities of 50:50:0.1 ACN:H₂O:TFA. All the solvents and matrix powders used were MS grade and purchased by Sigma Aldrich (Basel, Germany). ImagePrep matrix application method was in-house edited and tested in order to ensure a uniform coating and reduce analyte delocalization on the tissue.

MALDI-IMS analysis and data processing

MS experiments were performed on an Ultraflex III™ MALDI-TOF/TOF mass spectrometer (Bruker Daltonics) equipped with a Smartbeam™ Nd:YAG laser operating at 200 Hz. For all the imaging experiments, the MALDI source worked with ultra large laser diameter and used a laser attenuator at $65\% \pm 15\%$ in raster mode (spatial resolution 250 μ m), accumulating 400 laser shots for every position of the tissue. Full-MS spectra were acquired in linear TOF mode, positive ions, in a 3000–10 000 m/z range. Data analysis and processing was performed using the FlexAnalysis™ and FlexImaging™ software (Bruker Daltonics). All the spectra were firstly smoothed and baseline subtracted using the default FlexAnalysis™ algorithms, and then visualized with FlexImaging™ using normalization against TIC to better visualize the selected analytes.

Results and discussion

LTP isolation from tomato peel, pulp and seeds

Vine tomatoes of Piccadilly variety were used in order to detect the tomato LTP isoforms mainly present and to investigate their distribution across the three main fractions of tomato fruit (peel, pulp and seeds). Protein extraction was performed by means of precipitation with cold acetone, while pellet extraction was carried out in a phosphate buffered saline solution. The seeds were previously reduced to a fine powder by milling in liquid nitrogen. The quantification of protein extracts was performed by a bench fluorimeter, and the values obtained for each tissue were related to the amount of the dry material, as reported in Table 1.

Because LTPs are proteins with molecular weights ranging between 7 and 10 kDa, a simple and fast analytical method was developed, in order to concentrate the protein fraction containing them. A double ultracentrifugation step was carried out with each protein extract: the first one was performed by using centrifugal filter devices containing membranes with a nominal molecular weight limit (NMWL) of 30 kDa; the filtrated solutions, containing all the molecules with molecular weights lower than 30 kDa, were then applied to centrifugal filter devices containing membranes with an NMWL of 3 kDa. In this case, the retentates were recovered, as they contained proteins with molecular weight higher than 3 kDa. In this way, theoretically, all the molecules with masses included in the

MS data show that tomato LTP isoforms are in seeds

Table 1. Quantification of protein extracts from tomato peel, pulp and seed performed by Qubit® fluorometer

Tomato tissue	A_s (g)	A_{dm} (g)	P (mg)	Prot (%)
Pulp	50	0.9	29.48	3.27
Peel	10	2.04	78.82	3.86
Seed	6.37	3.07	94.67	3.08

A_s , amount of sample; A_{dm} , amount of dry sample; P , amount of extracted proteins, obtained by Qubit® fluorimeter; Prot %, percentage of proteins related to A_{dm} .

range 3–30 kDa were isolated from the total protein extract of each tomato tissue, concentrating the proteins of interest and at the same time excluding high molecular mass proteins and low molecular mass interferents, such as polyphenols. Although the membranes are characterized by an NMWL, because these limits were very far from the supposed molecular mass of LTP proteins (7–10 kDa), the possibilities that LTPs could be lost were very low.

Investigation of tomato extracts for tomato LTP discovery by UPLC-ESI-MS analysis

The content of protein fractionated extracts from tomato peel, pulp and seed was further investigated by UPLC coupled to an electrospray single quadrupole mass spectrometer (ESI-MS). The search of proteins of interest was carried out for every tomato protein fraction by investigating mass spectra relating to each chromatographic peak and identifying the molecular mass through the multicharged clusters of ions. In this way, four proteins with very close retention times and molecular weights similar to the ones of plant LTPs, that is, 7039 (± 1) Da, 9354 (± 1) Da, 9441 (± 1) Da and 9724 (± 1) Da, were detected only in the seed fraction (the isoform having Mw 9441 is reported as example in Fig. 1).

The retention times and the characteristic ions are summarized in Table 2.

As shown, all the four proteins had a molecular weight close to the one already found for other plant LTPs, such as Pru P 3 from peach (9135 Da),^[30] LTPa and LTPb from pear (9252 and 9250 Da),^[31] LTPa and LTPb from pomegranate (9342 and 9467 Da),^[32] LTP from maize (9046 Da),^[33] and Mal d 3 from apple (9076 Da).^[34] Quite interestingly, all the four potential tomato LTP isoforms co-eluted in two

Table 2. Retention times and characteristic ions of potential identified lipid-transfer protein in tomato seeds

Retention time (min)	Average mass (± 1 Da)	MS spectra characteristic ions (m/z)
25.5 26.4	7039	1760.5 (MH_4^{4+}) 1408.9 (MH_5^{5+}) 1174.1 (MH_6^{6+})
26.8 29.7	9354	1560.1 (MH_6^{6+}) 1337.1 (MH_7^{7+}) 1170.2 (MH_8^{8+})
26.6 29.9	9441	1889.2 (MH_5^{5+}) 1574.4 (MH_6^{6+}) 1349.6 (MH_7^{7+}) 1181.1 (MH_8^{8+})
25.6 27.7	9724	1945.9 (MH_5^{5+}) 1621.5 (MH_6^{6+}) 1390.1 (MH_7^{7+}) 1216.5 (MH_8^{8+})

chromatographic peaks (as shown in Fig. 1 for the 9441 kDa isoform), which had the same mass spectrum. It can be hypothesized that each isoform could undergo a partition between a folded conformation (or native), which is the first to elute, and a partially unfolded conformation (partially denatured), which interacts more with the stationary phase. Alternatively, it cannot be excluded that these proteins, having been isolated in their native state, partially carry a lipophilic molecule that alters the chromatographic retention time, albeit MS analysis did not show any evidence for this possible ligand. Further studies will be necessary in order to clarify this behaviour. Besides the molecular masses, the retention times of the extracted peaks were very similar to the one obtained for the pure peach LTP (data not shown), suggesting a similar protein. Quite interestingly, none of the molecular masses of these proteins fit with the masses of the tomato LTPs already known in literature (Table 3).

Structural characterization of potential tomato LTP isoforms

As no weight match was observed in the UniProtKB/SwissProt database for the potential detected tomato LTP isoforms, in order to obtain more information about the nature of these proteins and

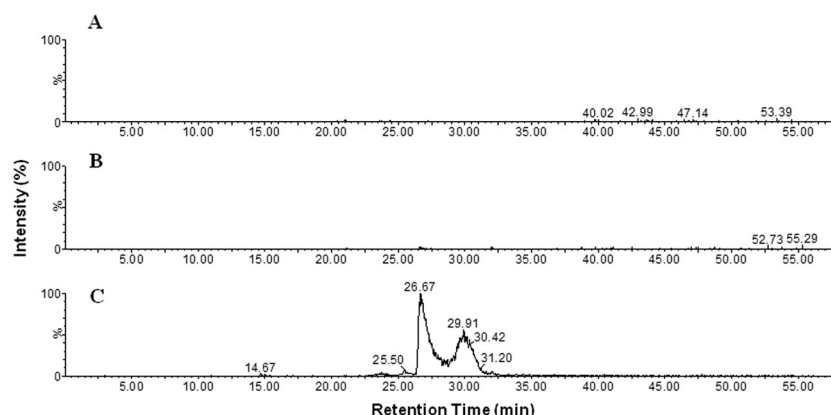


Figure 1. Example of extracted ion chromatograms for the protein having Mw 9441 Da obtained in tomato Piccadilly (A) peel, (B) pulp and (C) seed fractions by extracting from the full scan chromatogram the characteristic ions as reported in Table 2. The signals were normalized considering the highest intensity, that is, the one from seed fraction (e^8).

Table 3. List of all the tomato lipid-transfer protein (LTP) isoforms annotated in UniProtKB/SwissProt database and their corresponding molecular weights, as calculated by Proteomics ToolKit software^a without the predicted signal peptides and considering no amino acid modification

Accession number	Organism	Average mass (Da)	Mono isotopic mass (Da)
Q3YMR2 —Non-specific LTP 2	<i>Solanum chilense</i>	9210.6	9204.5
O24037 —Non-specific LTP 1	<i>Solanum pennellii</i>	9039.6	9033.6
O24038 —Non-specific LTP 2	<i>Solanum pennellii</i>	9185.6	9179.5
E2GLP6 —Non-specific LTP	<i>Solanum lycopersicum</i> var. <i>cerasiforme</i>	9381.1	9374.8
P27056 —Non-specific LTP 1	<i>Solanum lycopersicum</i>	9096.7	9090.7
P93224 —Non-specific LTP 2	<i>Solanum lycopersicum</i>	8943.3	8937.4
Q4A1N0 —Non-specific LTP	<i>Solanum lycopersicum</i>	8943.3	8937.4
Q4A1N1 —Non-specific LTP	<i>Solanum lycopersicum</i>	9379.9	9373.7

^a<http://db.systemsbiology.net:8080/proteomicsToolkit/index.html>.

to confirm their actual nature as LTP proteins, another investigation was carried out by exploiting the tertiary structure feature that is preserved among all the plant LTPs, that is, the four disulphide bridges holding together eight cysteine residues. The reduction and alkylation of these proteins with DTT and 2-iodoacetamide, in fact, would imply a shift in their molecular weight of 464 (± 1) Da, because of the link of a 58 Da carboxymethyl group to each cysteine residues. Thus, fractionated protein extracts from tomato peel, pulp and seeds, prepared as described earlier, were denatured by urea and then reduced by DTT, to break the disulphide bridges, and alkylated by 2-iodoacetamide. Afterwards, for each protein of interest, characteristic ions were inferred, considering the shift of mass due to reduction and alkylation; they were extracted from the TICs, and the multicharged pattern was investigated for each extract ion chromatogram (XIC). In this way, and once again only in seed fraction (the isoform having Mw 9906, corresponding to reduction and alkylation of the one having Mw 9441, is reported as an example in Fig. 2), four proteins with molecular masses corresponding to the previous ones with an increase of 464 (± 1) Da were found, and their retention times and characteristic ions are summarized in Table 4.

Assessment of protein identity by proteomic bottom-up approach

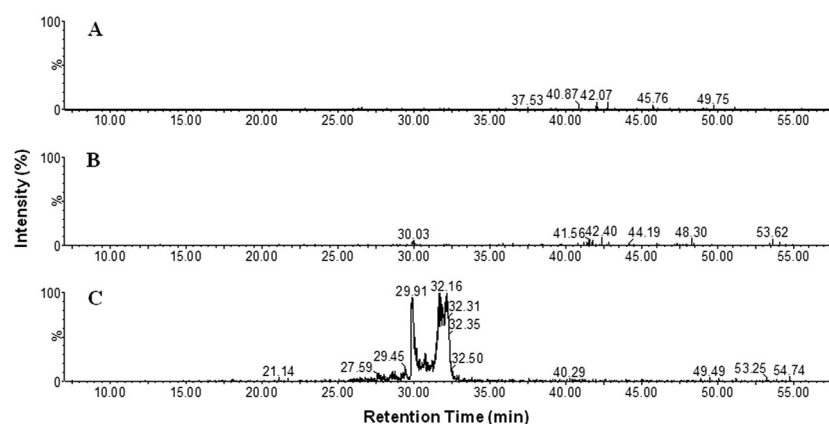
In order to obtain even more sequence information and definitively assess the nature of these potential tomato LTP isoforms, the fractionated extract from seeds was applied to a BioSep-Sec-S column

Table 4. Retention times and characteristic ions of reduced and alkylated potential lipid-transfer proteins in tomato seeds

Retention time (min)	Average mass (± 1 Da)	MS spectra characteristic ions (m/z)
28.1	7502	1501.1 (MH_5^{5+}) 1251.5 (MH_6^{6+}) 1072.9 (MH_7^{7+})
32.3	9818	1403.7 (MH_7^{7+}) 1228.2 (MH_8^{8+}) 1091.8 (MH_9^{9+})
29.9	9906	1415.8 (MH_7^{7+}) 1239.1 (MH_8^{8+})
32.1	10 189	1699.4 (MH_6^{6+}) 1456.5 (MH_7^{7+}) 1274.7 (MH_8^{8+})

for a gel filtration analysis, to have a better separation of the remaining proteins from those of interest (Fig. 3). SDS-PAGE identified the fractions containing proteins with the expected molecular weight (Fig. 3, panel B).

These were further analysed by UPLC-MS analysis, in order to detect the fractions really containing the potential isoforms already found before. Among these, fraction 12 showed the more intense signals related to the characteristic ions of the three 9 kDa proteins previously detected. On the contrary, no diagnostic ion for the

**Figure 2.** Example of extracted ion chromatograms for the protein having Mw 9906 Da, derived from the reduction and alkylation of the protein having Mw 9441 Da, obtained in tomato Piccadilly (A) peel, (B) pulp and (C) seed fractions by extracting from the full scan chromatogram the characteristic ions as reported in Table 4. The signals were normalized considering the highest intensity, that is, the one from seed fraction (e^7).

MS data show that tomato LTP isoforms are in seeds

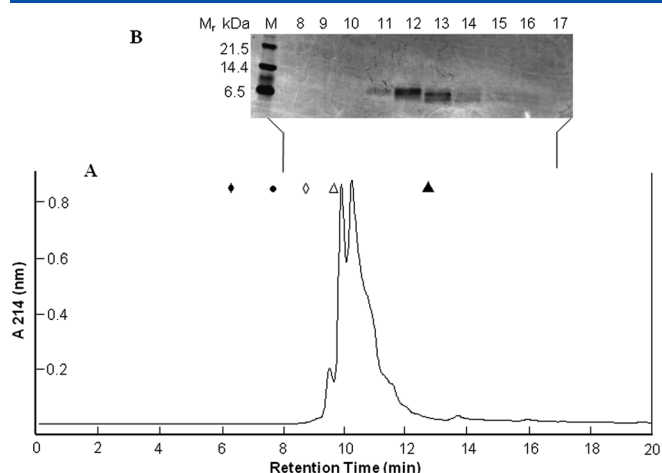


Figure 3. (A) Chromatogram related to the size exclusion chromatography of the lipid-transfer protein (LTP)-enriched extract from tomato seed. Molecular weight standards were as follows: ♦, bovine thyroglobulin (Mr 670 kDa); ●, bovine gamma globulin (Mr 158 kDa); ◇, chicken ovalbumin (Mr 44 kDa); △, horse myoglobin (Mr 17 kDa); ▲, vitamin B₁₂ (Mr 1.35 kDa). (B) Identification of LTP-containing fractions by SDS-PAGE analysis. Lane marked 'M' corresponds to molecular weight markers, and their relative molecular masses (M_r) are reported on the left side.

7 kDa potential LTP was detected, either in fraction 12 or in the following fractions, where the molecules with a lower molecular weight or a different folding were meant to be. For these reasons,

after a desalting step by ultracentrifugation, fraction 12 underwent an in-solution trypsin digestion. Analysis of the tryptic digested, performed by LTQ-Orbitrap, revealed the presence of two diagnostic peptides (Table 5) that, after a search of the UniProtKB/SwissProt databases against *Viridiplante* subset, were found to belong to nsLTPs from other species.

In particular, the former (CGVQLSVPIR, named here as peptide 1) was found to fully belong to kiwi's LTP isoforms (Uniprot accession nos. P86137 and P85206); the latter (CNCLK, here referred to as peptide 2) was found to be a common motif, included in larger tryptic peptides, in many plant LTPs, such as black mulberry (Uniprot accession no. P85894), plum (Uniprot accession no. P82534), peach (Uniprot accession no. P81402), apricot (Uniprot accession no. P81651), wheat (Uniprot accession no. P24296), apple (Uniprot accession no. Q9M5X7), pear (Uniprot accession no. Q9M5X6), kiwi (Uniprot accession nos. P86137 and P85206), chickpea (Uniprot accession no. O23758), rice (Uniprot accession no. Q2QYL2), cherry (Uniprot accession no. Q9M5X8), almond (Uniprot accession no. Q43019), barley (Uniprot accession no. P07597), lentil (Uniprot accession no. A0AT28), sorghum (Uniprot accession no. Q43193) and maize (Uniprot accession no. P19656). This finding definitely confirmed the identity of the identified proteins as LTP. On the other hand, a search of the already annotated tomato LTPs for these two motives did not find any perfect match, confirming that the LTP proteins here isolated did not match the tomato LTPs already present in the UniProtKB/SwissProt database.

Table 5. Diagnostic peptides, named peptide 1 and peptide 2, originated from the tryptic digestion of novel tomato lipid-transfer proteins (LTPs) in seed fraction and identified by LTQ-Orbitrap

Name	Sequence	Calculated Mw (Da) ^a	Observed Mw (Da)	MS spectra characteristic ions (m/z)
Peptide 1	C*GVQLSVPIR ^b	1214.62	1214.65	899.39 (y ₈), 472.26 (y ₄), 262.19 (y ₂)
Peptide 2	RTAC*NC*LK ^b	1021.44	1021.48	876.29 (b ₇), 763.23 (b ₆), 603.29 (b ₅), 489.22 (b ₄)

For peptide 2, the whole sequence containing the motif shared with several plant LTPs (CNCLK) has been reported (see the text for details).

^aMonoisotopic Mw.

^bC* indicates a carboxamidomethylcysteine.

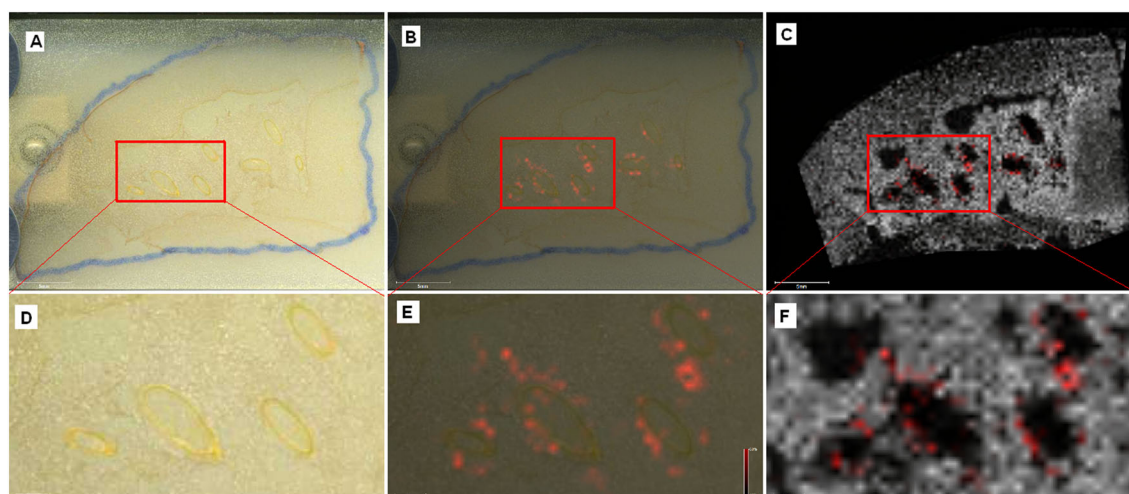


Figure 4. (A) Scanned image of Piccadilly tomato slice coated with MALDI matrix. (B) Extracted lipid-transfer protein signal at m/z 7039 $\pm$ 5 Da are localized around seeds only (red mark), even comparing to a well-represented signal of the pulp (white mark in panel C) at m/z 4366. Seeds are easily distinguished from pulp and peel in corresponding zoomed images D–F. In panel F, we can notice strong signal suppression in the seed area (black holes).

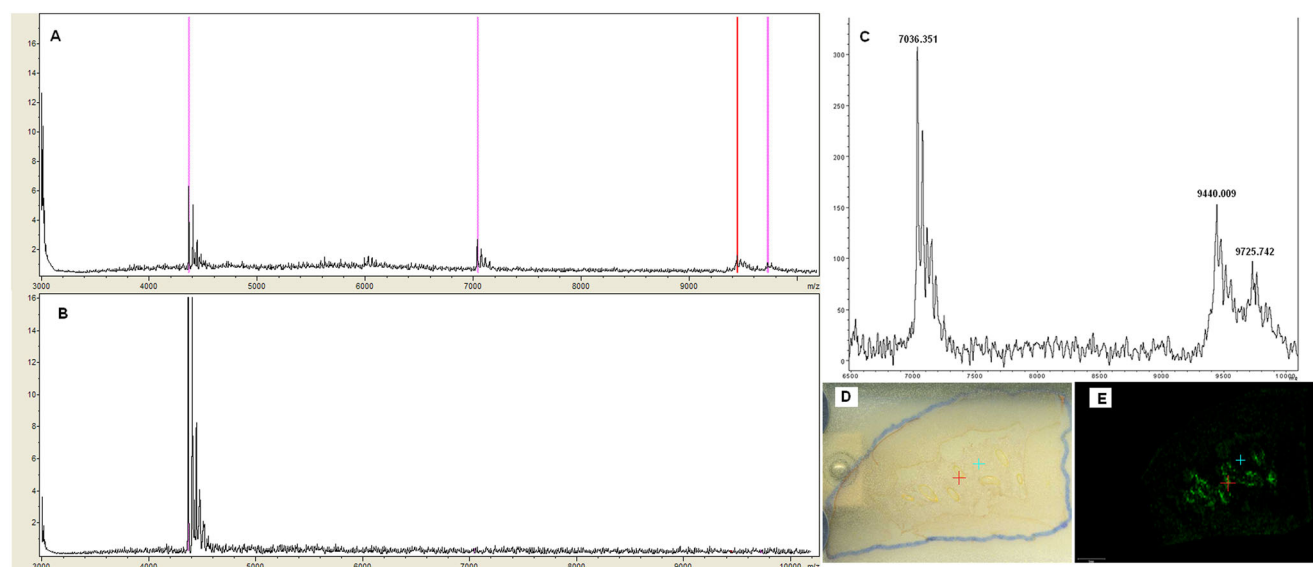


Figure 5. (A) Full-MS spectra taken directly on tissue (cross in panels D and E) showing lipid-transfer protein masses, compared with full-MS spectra (B) taken in the pulp area (cross in panels D and E). Scanned image (D) and merged MS image (E) of the three analytes clearly outlining their localization near the seeds.

LTP localization through MALDI imaging

Ions at m/z 7039, 9441 and 9724, corresponding to three of the four identified LTPs, were also identified by MALDI analysis, extracted in a window of 10 mass units and visualized with normalization against TIC. As shown in Fig. 4, the signal of one LTP at m/z 7039 exhibits a consistent increase in peak abundance in the regions close to the seeds, while in other zones of the slice, these mass peaks were not detected.

In Fig. 5, the average mass spectrum obtained by 400 laser shots on one seed (as evidenced also by the corresponding optical image) showed the three signals of interest compared with another mass spectrum taken in the pulp region, where these peaks are totally absent (also see Fig. 4C–F).

The small differences in mass values comparing ESI and MALDI mass spectra are probably due to the not null and not homogenous thickness of tomato slice in MALDI. From the zoomed MS image in Fig. 4, we also notice that the three molecules are mainly localized around seeds and not exactly in it; we were not able to explain if this different distribution between the zone near the surface of the seeds and the internal region of seeds is due to ion suppression or different extraction/crystallization between the two zones or it reflects a real different localization of these molecules between the outer and inner regions of the seeds.

Conclusions

All the presented data add further knowledge to the very complex scenario, as yet not fully understood, depicting the family of nsLTPs. The occurrence of so many LTP isoforms as found in tomato is not unusual across the plant kingdom: it is known, in fact, that in the three species for which the full genome sequence is available (rice, *Arabidopsis*, and poplar), approximately a dozen of the family LTP1 genes are present in each genome,^[35] but the reason for such a plethora of LTP isoforms is still unclear and probably related to their yet unknown biological role. Considering their hypothesized lipid binding capacity and their antimicrobial activity, for example, the primary structure of LTPs may need some arrangements, set by the evolutionary pressure, in order to adapt to the different environmental conditions. Also, the LTP distribution across different

plant tissues has been shown to be very variable. LTPs are concentrated in the peel of many plant species, especially in the Rosaceae family,^[36,37] but they have been detected also in the seeds^[38,39] and in the pulp^[23] of other plants, suggesting a possible correlation of their distribution with the genus and the species. Unlike what has been previously observed by Pravettoni and coworkers,^[23] in this study, tomato LTP isoforms have been found in the seeds, and no LTP isoform was revealed in peel and pulp tissues. Although this discrepancy might be due to low concentration of LTP, poor ionization yield or matrix effects, preventing us to detect the LTP in the other parts, there is no doubt that an effect of concentration close to seeds has been observed. This variability in LTP distribution may be related to the genotype or to the stage of ripening of the fruit, even if all the tomatoes analysed were bought in the market as mature for consumption. Also, LTP spatial and temporal expression, in turn, may depend on the functional and biological roles fulfilled by these proteins, which could be different according to the tissue, the developmental stages and the physiological conditions. Considering the enormous importance of tomato as food commodity in the world and the allergenic properties of LTPs, more studies investigating the presence of isoforms and their temporal and spatial distribution within the plant also during the ripening will be needed in the future, but it is undoubtedly clear that the elimination of the seed fraction will remove almost all the LTP content of a tomato product.

Acknowledgements

This study was supported by the Fondazione Cassa di Risparmio di Parma e Monte di Credito su Pegno di Busseto. Thanks to Mattia Geretti for his technical assistance in the preparation of protein extracts and fractionations, and to Barbara Prandi for assisting in setting up the chromatographical methods.

References

- [1] J. C. Kader. Proteins and the intra cellular exchange of lipids: I. Stimulation of phospholipid exchange between mitochondria and microsomal fractions by proteins isolated from potato tuber. *Biochim. Biophys. Acta* **1975**, 380, 31.

- [2] J. C. Kader. Lipid-transfer proteins in plants. *Annu. Rev. Plant. Physiol. Plant. Mol. Biol.* **1996**, 47, 627.
- [3] W. R. Bernhard, S. Thoma, J. Botella, C. R. Somerville. Isolation of a cDNA clone for spinach lipid transfer protein and evidence that the protein is synthesized by the secretory pathway. *Plant Physiol.* **1991**, 95, 164.
- [4] J. Pyee, H. Yu, P. E. Kolattukudy. Identification of a lipid transfer protein as the major protein in the surface wax of broccoli (*Brassica oleracea*) leaves. *Arch. Biochem. Biophys.* **1994**, 311, 460.
- [5] S. Thoma, U. Hecht, A. Kippers, J. Botella, S. De Vries, C. Somerville. Tissue-specific expression of a gene encoding a cell wall-localized lipid transfer protein from *Arabidopsis*. *Plant Physiol.* **1994**, 105, 35.
- [6] W. F. Broekaert, B. P. A. Cammue, M. F. C. De Bolle, K. Thevissen, G. W. De Samblanx, R. W. Osborn. Antimicrobial peptides from plants. *Crit. Rev. Plant Sci.* **1997**, 16, 297.
- [7] S. Jegou, J. P. Douliez, D. Molle, P. Boivin, D. Marion. Purification and structural characterization of LTP1 polypeptides from beer. *J. Agric. Food Chem.* **2000**, 48, 5023.
- [8] G. Salcedo, R. Sanchez-Monge, D. Barber, A. Diaz-Perales. Plant non-specific lipid transfer proteins: an interface between plant defence and human allergy. *Biochim. Biophys. Acta* **2007**, 1771, 781.
- [9] J. Gomar, P. Sodano, M. Ptak, F. Vovelle. Homology modelling of an antimicrobial protein, Ace-AMP1, from lipid transfer protein structures. *Fold. Des.* **1997**, 2, 183.
- [10] F. Pelèse-Siebenbourg, C. Caelles, J. C. Kader, M. Delseny, P. Puigdomènech. A pair of genes coding for lipid-transfer proteins in *Sorghum vulgare*. *Gene* **1994**, 148, 305.
- [11] V. Arondel, C. Vergnolle, C. Cantre, J. C. Kader. Lipid transfer proteins are encoded by a small multigene family in *Arabidopsis thaliana*. *Plant Sci.* **2000**, 157, 1.
- [12] P. Sterk, H. Booij, G. A. Scheleekens, A. Van Kammen, S. C. De Vries. Cell-specific expression of the carrot EP2 lipid transfer protein gene. *Plant Cell* **1991**, 3, 907.
- [13] L. Sossountzov, L. Ruiz-Avila, F. Vignols, A. Jolliot, V. Arondel, F. Tchang, M. Grosbois, F. Guerbette, E. Miginiac, M. Delseny, P. Puigdomènech, J. C. Kader. Spatial and temporal expression of a maize lipid transfer protein gene. *Plant Cell* **1991**, 3, 923.
- [14] L. Dubreil, T. Gaborit, B. Bouchet, D. J. Gallant, W. F. Broekaert, L. Quillien, D. Marion. Spatial and temporal distribution of the major isoforms of puroindolines (puroindoline-a and puroindoline-b) and non specific lipid transfer protein (ns-LTP1e1) of *Triticum aestivum* seeds. Relationships with their in vitro antifungal properties. *Plant Sci.* **1998**, 138, 121.
- [15] A. J. Fleming, T. Mandel, S. Fofmann, P. Sterk, S. C. de Vries, C. Kuhlemeier. Expression pattern of a tobacco lipid transfer protein gene within the shoot apex. *Plant J.* **1992**, 2, 855.
- [16] K. Gausing. Lipid transfer protein genes specifically expressed in barley leaves and coleoptiles. *Planta* **1994**, 192, 574.
- [17] F. Vignols, M. Wigger, J. M. Garcia-Garrido, F. Grellet, J. C. Kader, M. Delseny. Rice lipid transfer protein (LTP) genes belong to a complex multigene family and are differentially regulated. *Gene* **1997**, 195, 177.
- [18] S. Thoma, Y. Kaneko, C. Somerville. A non-specific lipid transfer protein from *Arabidopsis thaliana* is a cell wall protein. *Plant J.* **1993**, 3, 427.
- [19] A. L. Plant, A. Cohen, M. S. Moses, E. A. Bray. Nucleotide sequence and spatial expression pattern of a drought- and abscisic acid-induced gene of tomato. *Plant Physiol.* **1991**, 97, 900.
- [20] S. Torres-Schumann, J. A. Godoy, J. A. Pintor-Toro. A probable lipid transfer protein gene is induced by NaCl in stems of tomato plants. *Plant Mol. Biol.* **1992**, 18, 749.
- [21] M. B. Trevino, M. A. O'Connell. Three drought-responsive members of the non-specific lipid transfer protein gene family in *Lycopersicon pennellii* show different developmental patterns of expression. *Plant Physiol.* **1998**, 116, 1461.
- [22] L. Q. Le, Y. Lorenz, S. Scheurer, K. Fötisch, E. Enrique, J. Bartra, S. Biemelt, S. Vieths, U. Sonnewald. Design of tomato fruits with reduced allergenicity by dsRNAi-mediated inhibition of ns-LTP (Lyc e 3) expression. *Plant Biotechnol. J.* **2006**, 4, 231.
- [23] V. Pravettoni, L. Primavesi, L. Farioli, O. V. Brenna, C. Pompei, A. Conti, J. Scibilia, M. Piantanida, A. Mascheri, E. A. Pastorello. Tomato allergy: detection of IgE-binding lipid transfer proteins in tomato derivatives and in fresh tomato peel, pulp, and seeds. *J. Agric. Food Chem.* **2009**, 57, 10749.
- [24] R. Asero, G. Mistrello, S. Amato. Anaphylaxis caused by tomato lipid transfer protein. *Eur. Ann. Allergy Clin. Immunol.* **2011**, 43, 125.
- [25] L. Primavesi, V. Pravettoni, O. V. Brenna, L. Farioli, E. A. Pastorello, C. Pompei. Influence of technological processing on the allergenicity of tomato products. *Eur. Food Res. Technol.* **2011**, 232, 631.
- [26] M. Bencivenni, A. Faccini, C. Bottesini, R. Rao, A. Detoraki, E. Ridolo, G. Marone, P. P. Dall'Aglio, A. Dossena, R. Marchelli, S. Sforza. Assessing allergenicity of different tomato ecotypes by using pooled sera of allergic subjects: identification of the main allergens. *Eur. Food Res. Technol.* **2012**, 234, 405.
- [27] R. Lemarie, J. C. Tabet, P. Ducory J. B. Hendra. Solid ionic matrixes for direct tissue analysis and MALDI imaging. *Anal. Chem.* **2006**, 78, 809.
- [28] C. D. Calvano, S. Carulli, F. Palmisano. Aniline/a-cyano-4-hydroxycinnamic acid is a highly versatile ionic liquid for matrix-assisted laser desorption/ionization mass spectrometry. *Rapid Comm. Mass Spec.* **2009**, 23, 1659.
- [29] E. Giménez, F. Benavente, J. Barbosa, V. Sanz-Nebot. Ionic liquid matrices for MALDI-TOF-MS analysis of intact glycoproteins. *Anal. Bioanal. Chem.* **2010**, 398, 357.
- [30] V. Cavatorta, S. Sforza, G. Mastrobuoni, G. Pieraccini, S. Francese, G. Moneti, A. Dossena, E. A. Pastorello, R. Marchelli. Unambiguous characterization and tissue localization of Pru P 3 peach allergen by electrospray mass spectrometry and MALDI imaging. *J. Mass Spectrom.* **2009**, 44, 891.
- [31] I. Ramazzina, S. Amato, E. Passera, S. Sforza, G. Mistrello, R. Berni C. Folli. Isoform identification, recombinant production and characterization of the allergen lipid transfer protein 1 from pear (Pyr c 3). *Gene* **2012**, 491, 173.
- [32] G. Zoccatelli, C. Dalla Pellegrina, M. Consolini, M. Fusi, S. Sforza, G. Aquino, A. Dossena, R. Chignola, A. Peruffo, M. Olivieri, C. Rizzi. Isolation and identification of two lipid transfer proteins in pomegranate (*Punica granatum*). *J. Agric. Food Chem.* **2007**, 55, 11057.
- [33] K. Kuppannan, D. R. Albers, B. W. Schafer, D. Dielman, S. A. Young. Quantification and characterization of maize lipid transfer protein, a food allergen, by liquid chromatography with ultraviolet and mass spectrometric detection. *Anal. Chem.* **2011**, 83, 516.
- [34] A. I. Sancho, N. M. Rigby, L. Zuidmeer, R. Asero, G. Mistrello, S. Amato, E. González-Mancebo, M. Fernández-Rivas, R. van Ree, E. N. Mills. The effect of thermal processing on the IgE reactivity of the non-specific lipid transfer protein from apple, Mal d 3. *Allergy* **2005**, 60, 1262.
- [35] T. H. Yeats, J. K. C. Rose. The biochemistry and biology of extracellular plant lipid transfer proteins (LTPs). *Prot. Sci.* **2008**, 17, 191.
- [36] A. Diaz-Perales, G. Garcia-Casado, R. Sanchez-Monge, F. J. Garcia-Salles, D. Barber, C. Salcedo. cDNA cloning and heterologous expression of the major allergens from peach and apple belonging to the lipid-transfer protein family. *Clin. Exp. Allergy* **2002**, 32, 87.
- [37] G. Marzban, H. Puehringer, R. Deya, S. Brynda. Localisation and distribution of the major allergens in apple fruits. *Plant Sci.* **2005**, 169, 387.
- [38] N. M. Rigby, J. Marsh, A. I. Sancho, K. Wellner, J. Akkerdaas, R. van Ree, A. Knulst, M. Fernández-Rivas, V. Brettlova, P. P. Schilte, C. Summer, R. Humphrey, P. R. Shewry, E. N. Mills. The purification and characterisation of allergenic hazelnut seed proteins. *Mol. Nutr. Food Res.* **2008**, 52, 251.
- [39] L. P. Cruz, S. F. Ribeiro, A. O. Carvalho, I. M. Vasconcelos, R. Rodrigues, M. Da Cunha, V. M. Gomes. Isolation and partial characterization of a novel lipid-transfer protein (LTP) and antifungal activity of peptides from chilli pepper seeds. *Protein Pept. Lett.* **2010**, 17, 311.