

Lipid-transfer proteins are relevant allergens in fruit allergy

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Background: Allergy to apple and *Prunus* fruits is frequently associated with birch pollinosis, with the principal cross-reacting allergens involved being members of the Bet v 1 family. However, a major 13-kd component, nonimmunologically related to Bet v 1, has been implicated as allergen in patients allergic to *Prunoideae* fruit but not to birch pollen.

Objective: We sought to isolate and characterize the 13-kd allergen present in apple and peach.

Methods: Sera from patients allergic to both fruits were selected on the basis of clinical symptoms, skin prick tests responses, and specific IgE levels. Allergens were purified by reverse-phase HPLC and characterized by N-terminal amino acid sequencing, MALDI analysis, specific IgE immunodetection, and immunoblot inhibition assays.

Results: A 13-kd protein band was recognized in crude apple and peach extracts by 9 of 10 and 10 of 10 sera from patients allergic to fruit, respectively. The isolation and characterization of the corresponding allergens allowed their identification as lipid-transfer proteins, with a molecular mass of 9058 d for the apple protein and 9138 d for the peach protein. Both purified allergens were recognized by sera from patients allergic to fruit and fully inhibited the IgE binding by the 13-kd component present in the 2 crude fruit extracts.

Conclusion: Lipid-transfer proteins are relevant apple and peach allergens and, considering their ubiquitous distribution in tissues of many plant species, could be a novel type of panallergen of fruits and vegetables. (*J Allergy Clin Immunol* 1999;103:514-9.)

Key words: Apple, peach, allergen, lipid-transfer proteins, IgE binding

Allergy to apple, as well as to other plant foods such as cherry, celery, and carrot, is commonly associated with birch pollinosis in areas where birch trees are abundant.¹⁻³ This cross-sensitization has been mainly explained by the presence of homologous allergens in both the pollen from trees of the *Fagales* order and the fruits/vegetables mentioned above. Thus 16- to 18-kd proteins that share common IgE epitopes with Bet v 1, the major birch pollen

Abbreviations used

LTP:	Lipid-transfer protein
MALDI:	Matrix-assisted laser desorption/ionization mass spectrometry
MW:	Molecular weight

allergen,⁴ have been characterized in alder, hazel, and hornbeam pollen⁵ and in celery, cherry, and carrot.⁶⁻⁸ Mal d 1, the only apple allergen isolated so far, belongs to this allergen group (63% amino acid sequence identity with Bet v 1).⁹ Besides the Bet v 1 protein family, profilins^{10,11} and isoflavone reductases¹² are also panallergens that seem to be involved in cross-reactions between birch pollen and plant foods.

In contrast to this association between allergy to fruits/vegetables and allergy to tree pollens, several cases of hypersensitivity to peach and related fruits, but with negative responses to pollens, have been described.¹³ In these cases a major 13-kd IgE-binding protein, which is not inhibited by grass or birch pollen extract in immunoblot inhibition assays, has been detected.¹³ This low molecular weight (MW) allergen is present in peach, apricot, plum, cherry, and probably mulberry¹³⁻¹⁵ and seems to be confined to the fruit skin (at least in peach).¹⁴ The characterization of the 13-kd component and its relationship with other plant proteins remains unpublished.

We report here the isolation and characterization of the low MW allergens from apple and peach and their identification as lipid-transfer proteins. The ubiquitous distribution of this protein family in a variety of tissues from many plant species¹⁶ suggests its potential role as a panallergen in plant foods.

METHODS

Patient sera

Sera from 10 patients allergic to apple and peach were used in this study. All patients showed immediate hypersensitivity to both fruits according to their clinical history, positive response to skin prick tests with the corresponding fruit extracts (ALK-Abelló, Madrid, Spain), and specific IgE levels to apple and peach greater than 1.4 kU/L. Clinical data of the 10 selected patients are summarized in Table I. In addition, sera from 3 subjects not sensitized to fruits (negative skin prick test responses and undetectable specific IgE to apple, peach, and plum), but with total serum IgE levels similar to those of the patients allergic to fruit, were used as negative controls. Written informed consent from the selected patients and control subjects was obtained.

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Supported by Dirección General de Enseñanza Superior, MEC (grant PB95-0035).

Received for publication Aug 6, 1998; revised Sept 24, 1998; accepted for publication Sept 25, 1998.

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0091-6749/99 \$8.00 + 0 1/1/94901

Total and specific IgE levels were quantified by the Pharmacia CAP System FEIA (Pharmacia Diagnostics, Uppsala, Sweden). Skin prick tests were performed with commercial glycerinated preparations (ALK-Abelló) by conventional procedures, and wheal areas at least 5 mm² greater than that produced by the saline control were considered positive.

Crude extract preparation

Source materials supplied by ALK-Abelló, which consisted of ground skins of apple (*Malus domestica*) or peach (*Prunus persica*), were used as starting material for allergen purification. These preparations were first extracted with PBS buffer (1:10 wt/vol) for 1 hour at 4°C. After centrifugation (10,000 rpm for 25 minutes at 4°C), the supernatants were dialyzed against H₂O and freeze-dried. The material obtained was then subjected to gel-filtration chromatography on an Econo-Pac P6 desalination column (5 mL of total volume; Bio-Rad, Hercules, Calif) by using 0.1 mol/L ammonium acetate as elution buffer. The appropriate fractions corresponding to the protein peak were pooled and freeze-dried (crude extracts).

Isolation and characterization of allergens

The crude extracts were defatted with cold acetone and then with ethanol:ether (1:3 vol/vol) (1:5 wt/vol for 1 hour at 4°C). The dried residues were fractionated by reverse-phase HPLC (RP-HPLC) on a preparative Vydac-C4 column (22 × 250 mm; particle size 10 µm; The Separations Group, Hesperia, Calif). Elution was carried out with a 2-step linear gradient of acetonitrile in 0.1% trifluoroacetic acid (0% to 40% in 120 minutes and 40% to 85% in 30 minutes; 1 mL/min) after washing for 15 minutes with 0.1% trifluoroacetic acid. The peak corresponding to the allergen was identified by SDS-PAGE and immunodetection of the RP-HPLC fractions.

SDS-PAGE was performed on 4% to 20% precast polyacrylamide gels (Bio-Rad), following the method of Laemmli.¹⁷ N-terminal amino acid sequences were determined by standard methods with an Applied Biosystems 477A gas-phase sequencer. Matrix-assisted laser desorption/ionization (MALDI) mass spectrometry analysis was carried out by using a MALDI-2 spectrometer with delayed extraction (Kratos Analytical, Manchester, UK). Protein concentration was determined according to the method of Bradford.¹⁸

Immunodetection and immunoblot inhibition assays

Samples were separated by SDS-PAGE on a 10% to 20% tricine precast polyacrylamide gel (Novex, San Diego, Calif) by using nonreducing conditions and then electrotransferred onto nitrocellulose membranes as described by Towbin et al.¹⁹ After blocking with 5% BSA in PBS for 2 hours at room temperature, the blots were incubated overnight with each individual serum (1:10 dilution in 0.5% BSA, 0.05% Tween-20 in PBS). After washing 4 times with 0.1% Tween-20 in PBS, membranes were overlaid with mouse anti-human IgE mAb HE-2 ascitic fluid²⁰ (1:3000 dilution) for 2 hours and then washed and incubated with a rabbit anti-mouse IgE peroxidase-conjugated antibody (DAKO A/S, Denmark) for 1 hour. Detection of IgE-binding components was achieved by enhanced chemiluminescence, following the manufacturer's instructions (ECL-Amersham). Sera from patients not sensitized to fruit, as well as dilution buffer, were used as negative controls.

Immunoblot inhibition assays were performed by using a pool of sera from patients 1 to 10 (Table I). Samples (10 µg protein of the appropriate crude extract) were separated by SDS-PAGE and electrotransferred onto nitrocellulose membranes. The serum pool diluted 1:15 was preincubated with each inhibitor (4 µg of purified aller-

gen or Der p 1 as negative control, in a final volume of 1 mL) for 1 hour at room temperature before exposure to the membranes.

RESULTS

Crude extracts of apple and peach were obtained by PBS buffer extraction of ground skins, followed by gel-filtration chromatography (see the Methods section). Protein content of the initial PBS extracts was 2.8% for apple and 1.5% for peach. Sera from 10 patients allergic to both fruits (Table I) were tested individually by specific IgE immunodetection after SDS-PAGE of the crude extracts (Fig 1). Several IgE-binding bands, ranging from around 6 to 100 kd, were detected, and the recognition patterns obtained with each serum were different. A component with a MW of about 13 kd was recognized in the apple extract by 9 of 10 sera (90%) and in the peach preparation by all 10 sera (100%), although the intensity of the corresponding band varied widely, depending on the serum assayed (compare lanes 4 and 5 in Fig 1, A).

To isolate the 13-kd allergen from both fruits, the corresponding crude extracts were defatted and then fractionated by RP-HPLC (Fig 2, A and B). The peak with the allergen was identified by SDS-PAGE and IgE immunodetection of the RP-HPLC fractions. Final yields of the isolated apple and peach allergens were 0.9% and 3.2% of the PBS-extracted protein, respectively. Homogeneity of the purified apple and peach proteins was checked by SDS-PAGE, during which both allergens showed a single band of around 13 kd, and by MALDI analysis, which revealed a single sharp peak in the 8 to 10 kd range, with a molecular mass of 9058 d for the apple protein and 9138 d for the peach protein.

To further characterize the isolated allergens, their N-terminal amino acid sequences were determined (Fig 3). No heterogeneity was detected at any position of the sequences obtained. The undetermined residues most probably corresponded to cysteines, considering that sequencing was carried out with native proteins. Both purified allergens, provisionally named Ma (the one from apple) and Pr (that from peach), had very similar primary structures, showing only 3 differences in the first 22 residues. Interestingly, they shared a high degree of sequence identity with several lipid-transfer proteins (LTPs) previously reported, such as those from almond (27 of 31 residues identical to Ma) and grape (19 of 31 identical to Ma) (Fig 3). These results allowed the identification of Ma and Pr as typical LTPs present in apple and peach, respectively. An intermediate similarity (10 of 31) was found between Ma and an allergen from *Parietaria judaica* pollen,²² which has been described as an LTP also. Conversely, a lower similarity (7 of 31) was found with a putative allergen from *Brassica rapa* pollen,²³ which belongs to an atypical subgroup of LTPs.

Either Ma or Pr was recognized when tested with a pool of sera from the 10 patients allergic to fruit (Fig 4). Furthermore, both of the allergens inhibited the IgE binding to the 13-kd band of the corresponding crude extract in immunoblot inhibition assays. Additionally, Pr was also able to fully inhibit the recognition by IgE of the 13-kd apple band (Ma) (Fig 4). No effect was exerted by the nonrelated

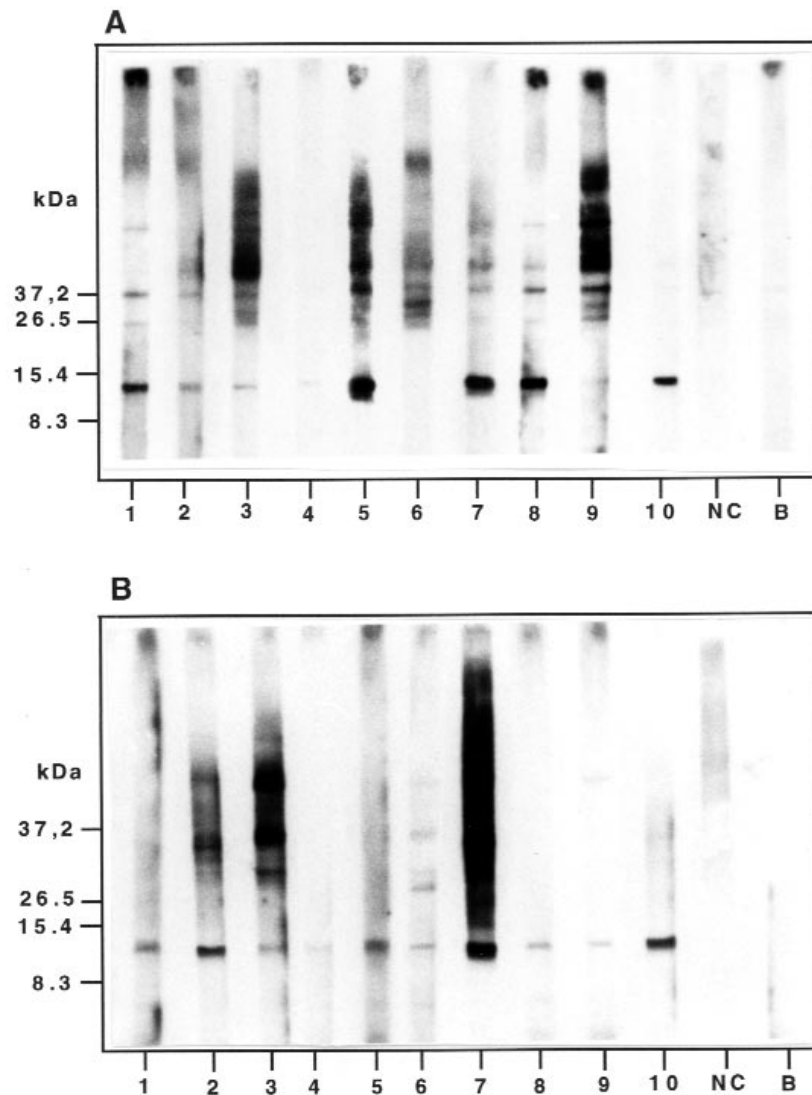


FIG 1. Immunoblot analysis of crude extracts from apple (**A**) and peach (**B**). Samples (10 μ g protein) were separated by SDS-PAGE, electrotransferred onto nitrocellulose membranes, and immunoblotted with individual sera from patients allergic to fruit (lanes 1 to 10), serum from a patient not allergic to fruit (NC), and dilution buffer (B).

TABLE I. Clinical data of the patients allergic to apple and peach

Patient	Age (y)	Sex	Symptoms	Total IgE (kU/L)	Specific IgE (kU/L)		SPT (mm ²)*	
					Apple	Peach	Apple	Peach
1	26	M	U,FA,GA	192	5.6	8.5	64	79
2	23	F	U,FA	481	10.5	17.6	95	128
3	24	F	U,FA,TA	760	26.2	32.2	79	64
4	27	M	U	355	2.0	2.4	33	38
5	33	M	U,FA	109	13.5	12.1	38	44
6	20	F	U,FA	262	1.7	1.4	79	41
7	25	M	U,FA	236	49.1	52.1	75	50
8	24	F	U,TA,V,A	368	4.1	6.1	16	13
9	22	M	FA	298	2.4	3.4	41	44
10	19	M	U,FA	64	20.2	17.8	44	57

U, Urticaria; FA, facial angioedema; GA, glottis edema; TA, tongue edema; V, vomiting; A, asthma.

*Skin prick tests expressed as wheal area.

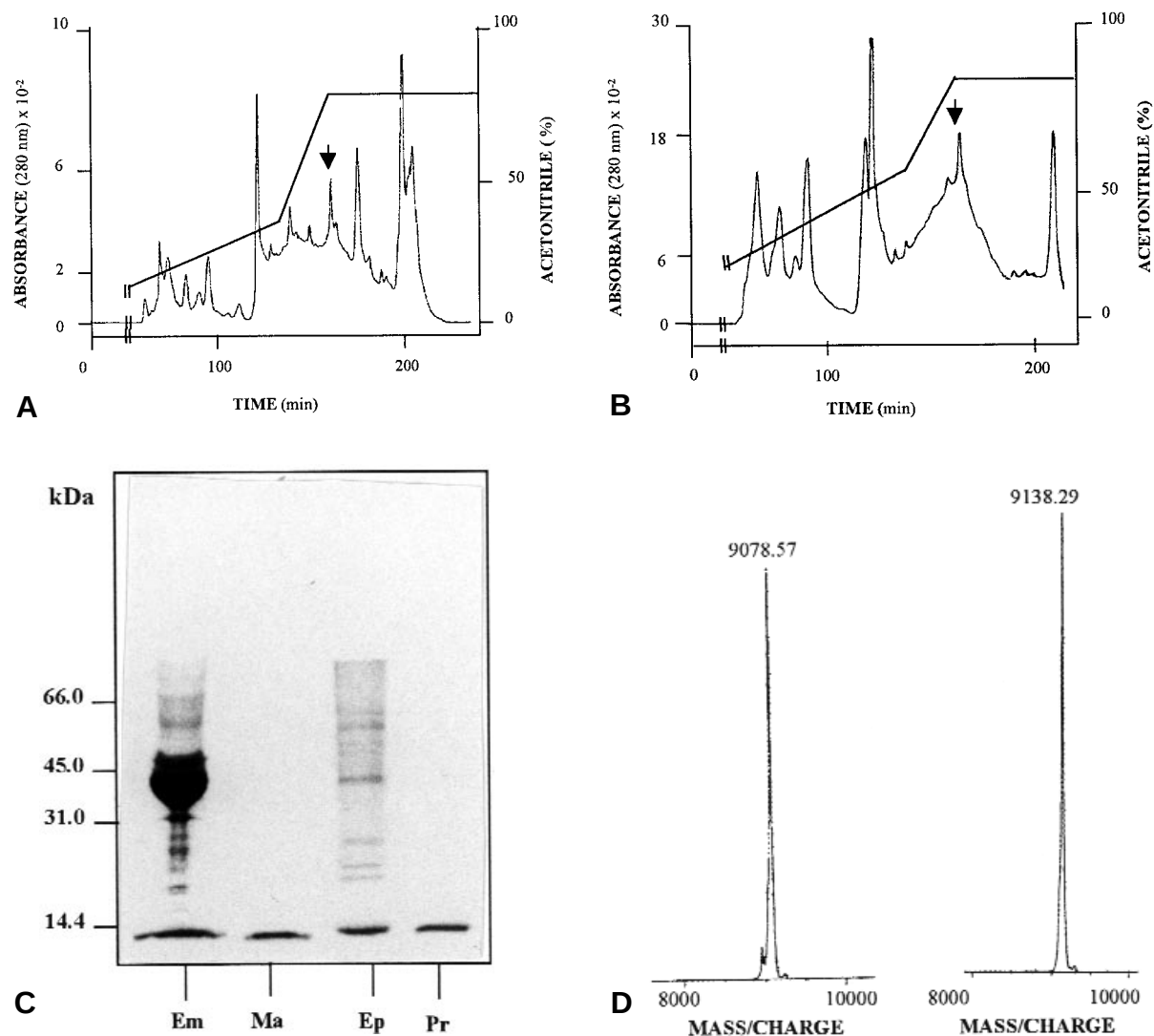


FIG 2. RP-HPLC fractionation of defatted crude extracts from apple (**A**) and peach (**B**). Arrow heads point to position of the isolated allergen in each chromatographic profile. **C**, SDS-PAGE separation of crude extract from apple (*Em*) and peach (*Ep*) and of the purified allergens from both fruits (*Ma* and *Pr*). **D** and **E**, MALDI analysis of the isolated *Ma* and *Pr* proteins, respectively.

PROTEIN	N-TERMINAL AMINO ACID SEQUENCE			
	1	10	20	30
Ma	ITXGQVTSSLAPXIGYVRS GGAVPPAXXNGI			
Pr	ITXGQVSSSLAPXIPYVRGGGA			
PALTP1	ITCGQVSSNLAPCIPYVRGGGAVPPACCNGI			
MLT2-VIT	ITCGQVSSALSSCLGYLKN GGAVPPGSSCGI			
Par j 1.0102	ETCGTVVRALMPCLPFVQGKEKEPSKGCCSG			
PEC-1	VEAACDPKQLQPCLAAITGGGQPSGDCCA KL			

FIG 3. N-terminal amino acid sequences of the allergens purified from apple (*Ma*) and peach (*Pr*) aligned with those previously reported for LTPs from almond (*PALTP1*; M Suelves, unpublished; EMBL accession number X96714), grape (*MLT2-VIT*),²¹ *Parietaria judaica* (*Par j 1.0102*),²² and *Brassica rapa* (*PEC-1*).²³

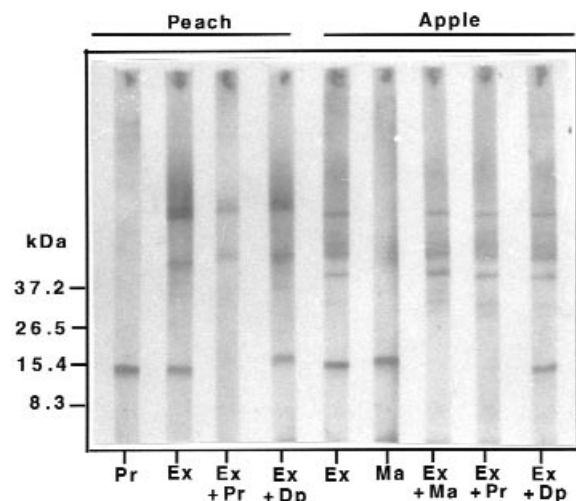


FIG 4. Immunoblot analysis of crude extracts (*Ex*) from peach and apple (10 μ g of protein) and the purified Pr and Ma allergens (1.3 μ g of protein). Samples were separated by SDS-PAGE, electrotransferred onto nitrocellulose membranes, and immunodetected with a pool of 10 sera from patients allergic to fruit. In immunoblot inhibition assays the serum pool was preincubated with 4 μ g each of Pr (*Ex + Pr*), Ma (*Ex + Ma*), or Der p 1 (*Ex + Dp*) allergens.

allergen Der p 1 from the mite *Dermatophagoides pteronyssinus* in similar inhibition assays.

DISCUSSION

Birch pollinosis is frequently associated with apple allergy in areas such as Central and Northern Europe, the United States, and Canada where trees belonging to the *Fagales* family are abundant. Although several allergens with structural similarities present in both pollen and fruit have been claimed to be responsible for this cross-sensitization,³ Mal d 1, the 17-kd apple protein homologous to Bet v 1, seems to be the most prominent cross-reacting allergen of this fruit in subjects allergic to both birch pollen and apple (70% to 90% of Mal d 1–positive patients).²⁴ We have proved the presence of the corresponding 17-kd IgE-binding band in the crude apple extract by testing it with a serum pool from patients allergic to birch pollen (not shown).

However, the population of patients allergic to *Rosaceae* fruits whose sera were assayed in this study lived in areas basically free of birch and other *Fagales* trees and therefore cannot be directly sensitized to these tree pollens. In fact, most of the 10 sera used (6 of 10) showed undetectable levels of specific IgE to birch pollen. In this type of allergic population, which is frequent in the Mediterranean countries, a major 13-kd allergen present in various *Rosaceae* fruits has been detected.^{13–15} Our results fully confirmed the relevance of this low MW allergen because a band of similar MW, as estimated by SDS-PAGE, was recognized in both apple and peach extracts by at least 9 of 10 positive sera tested (Fig 1).

The allergen was isolated from fruit skin preparations that, at least in peach, are highly enriched in the low MW IgE-binding band.¹⁴ The purified apple Ma and peach Pr proteins behaved as a single SDS-PAGE (denaturing conditions) band with the expected MW (around 13 kd), but MALDI analysis of these proteins revealed a single sharp peak of about 9.1 kd. Both isolated Ma and Pr retained their IgE-binding capacity and fully inhibited the recognition by IgE of the 13-kd component of the crude apple and peach extracts (Fig 4). These results indicate a cross-reactivity between Ma and Pr, allowing the presence of unrelated allergic proteins with similar MWs in the fruit preparations tested to be ruled out.

The N-terminal sequencing of the purified apple and peach allergens (31 residues for Ma, which represent about one third of its complete primary structure) has allowed confirmation of their close similarity. Moreover, it has led to disclosure of their relationship with plant LTPs, particularly with those from almond and grapevine (>80% and 60% of sequence identity when compared with Ma, respectively). An intermediate homology (about 32%) was found with the *Parietaria* allergen Par j 1.0102, which has been claimed to be an LTP.²² Surprisingly, a weak and even doubtful sequence relationship is shared with a pollen protein from *Brassica rapa*.²³ The uncertain inclusion of this putative *Brassica* allergen in the true plant LTP family, on the basis of its sequence characteristics,^{16,23} can account for the differences with Ma and Pr.

The extent of allergen characterization in *Rosaceae* fruits is still very limited. Besides Mal d 1, only a 31-kd allergenic protein homologous to wheat trimitin and oat avematin has been identified in apple.²⁵ No complete or partial sequence of a peach allergen has been published yet, although Pru a 1 (Bet v 1 homologous)⁶ and pru a 2 (a 23-kd thaumatin-like protein)²⁶ have been characterized in cherry (*Prunus avium*), a closely related species. Taking into account these designations and considering the relevance of Ma and Pr in *Rosaceae* fruit allergy, we suggest renaming both as Mal d 3 and Pru p 3, respectively.

Plant LTPs form a family of 9-kd (90 to 95 amino acids) polypeptides that is widely distributed throughout the plant kingdom. Different degrees of sequence similarity have been found between LTPs, most of which contain 8 conserved cysteines forming 4 disulfide bridges.¹⁶ The 3-dimensional structure of some LTPs has been determined, and a general model has been postulated with 4 α -helices and a C-terminal tail, with the overall structure stabilized by the 4 disulfide bridges. An internal hydrophobic cavity running through the molecule that can harbor lipidic components, such as fatty acids, is also present.¹⁶ It was originally proposed that LTPs play a role in the intracellular trafficking of lipids, but this hypothesis has now been largely abandoned because it is not consistent with several properties of this group of polypeptides, mainly its extracellular location. By contrast, LTPs are truly involved in the defense mechanisms of plants against pathogens and probably also participate in the formation of extracellular lipophilic materials, such as cutin or wax.^{16,27} Many of them are

located in the outer epidermal layer of the exposed surfaces of the plant, which is in line with the accumulation of Pru p 3 in the fruit skin.¹⁴ Several LTP genes are under developmental control and respond to pathogen infection, biotic stress conditions, or both.¹⁶ Some of these characteristics, together with the identification of Mal d 3 and Pru p 3 as LTPs, allow several considerations. Thus the wide distribution of LTPs among mono- and dicotyledonous species, suggests a potential role of this protein family as panallergens of plant foods. Moreover, substantial changes in the amount of LTPs (allergens) could be expected during fruit ripening, depending on environmental factors or storage conditions. Finally, the potential allergenicity of this protein family should be considered when LTP genes are used to obtain transgenic plants with enhanced resistance against pathogens.

We thank Dr Antonio Fernandez (ETSIA, UPM) for MALDI analysis, Joaquín García-Guijarro and Dolores Lamóneda (ETSIA, UPM) for technical assistance, Javier Varela (CIB, CSIC) for protein sequencing, and Linda Puebla (ALK-Abelló) for language revision.

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