

Original article

Lettuce anaphylaxis: identification of a lipid transfer protein as the major allergen

Background: Allergy to plant-derived foods is associated with birch pollinosis in central and northern Europe. Symptoms elicited are usually limited to the oropharyngeal system. By contrast, in the Mediterranean area, allergy to the same foods manifests more frequently with systemic reactions caused by nonspecific lipid transfer proteins (nsLTP), independently of an associated pollinosis.

Objective: We sought to investigate the pattern of immunoglobulin E (IgE) binding protein bands implicated in lettuce allergy, in particular the presence of an nsLTP.

Methods: Consecutive lettuce allergic patients were selected. Determination of serum-specific IgE, immunoblot, and inhibition experiments were performed in order to study the pattern of IgE binding proteins and the potential cross-reactivity to pollens. Inhibition studies with recombinant allergens were conducted to identify the lettuce allergens. The major allergen was subjected to N-terminal amino acid sequencing.

Results: Fourteen patients were diagnosed as being allergic to lettuce. All were sensitized to *Platanus* pollen. Ten of them showed specific IgE to a lettuce protein of 9-kDa. The IgE binding to this protein was completely inhibited by the cherry-LTP and peach extract. The N-terminal sequence of the 9-kDa protein showed a high degree of amino acid sequence identity to other nsLTPs. A clear partial cross-reactivity was observed between lettuce-LTP and *Platanus*-pollen extract.

Conclusions: An LTP has been demonstrated to be a major allergen in patients suffering from lettuce allergy.

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Allergy to foods of plant origin is frequently caused by pollen-vegetable food cross-reactive Immunoglobulin (IgE). Symptoms elicited by plant-derived food allergens are usually limited to the oropharyngeal system (1,2), most likely because of the lability of the allergens to digestion in the gastrointestinal tract (3). In central and northern Europe food allergy to plant-derived food is strongly associated with birch pollinosis because of the existence of IgE cross-reactive homologous allergens in birch pollen and food (4). By contrast, in the Mediterranean area, allergic reactions to the same plant-derived food are not related to birch pollen allergy, and are predominantly elicited by nonspecific lipid transfer proteins (nsLTPs) (5–10).

The LTPs are a class of conserved plant defense-related proteins which belong to the PR-14 group of pathogenesis-related proteins (11). They are small molecules of approximately 9–10 kDa that demonstrate high stability and, because of their high resistance to pepsin digestion, they are potentially severe food allergens (8).

The LTP is a pan-allergen with a degree of cross-reactivity comparable with profilin. Sensitization to profilin has been shown to be caused by exposure to pollen leading to IgE cross-reactivity to profilins in foods. In contrast, IgE reactivity to LTPs has been reported to occur not exclusively in pollen-allergic subjects, thereby suggesting that sensitization occurs by the oral route (12, 13).

To explain the differences in the pattern of reactivity between northern and southern European allergic patients, the existence of LTPs in pollen predominantly grown in the Mediterranean area has been studied. For example, ragweed pollen, and pollen of *Parietaria judaica* and mugwort have been found to contain proteins with homology to nsLTPs (8, 9, 14), but the potential role of a primary sensitization to these pollens as cause of cross-reactive food allergies remains unclear. So far, only partial cross-reactivity of food LTP-specific IgE with pollen LTP has been found (9).

Recently, the association of *Platanus* pollinosis and plant-derived food allergy in the Mediterranean area has

been reported (15, 16). Food allergens most frequently implicated were nuts, fruits (peach, apple, melon and kiwi), legumes and vegetables such as lettuce and green beans. Most of the patients with food allergy-related symptoms suffered from systemic reactions which suggest the presence of an LTP or another stable food allergen being responsible for IgE cross-reactions between *Platanus* pollen and plant-derived food (16).

Lettuce (*Lactuca sativa*) is a vegetable belonging to the *Compositae* family, in which other vegetables, such as endives, chicory and artichokes are included, as well as plants such as *Artemisia* (mugwort) and *Ambrosia* (ragweed), and others (17). Allergic reactions to lettuce are not very frequently reported. It has been identified mainly as a cause of occupational contact dermatitis (18–23). In last years, very few case reports of systemic reactions after its ingestion have been published (17, 24–28). Only in some of these reports the IgE binding components of lettuce extracts have been investigated showing IgE binding protein bands between 14 and 100 kDa (17, 24, 27, 28). To our knowledge, the presence of nsLTP in lettuce has not been previously reported.

In this study, we describe 14 patients who suffered from allergic reactions after lettuce ingestion. We studied the pattern of IgE binding protein bands, and the presence of an LTP responsible for these reactions. As an association with *Platanus* pollinosis in the Mediterranean area has been suggested (16), potential cross-reactivity to pollen was investigated by immunoblotting inhibition experiments.

Methods

Patients

Consecutive patients diagnosed of lettuce hypersensitivity at the Allergy Department of the Institut Universitari Dexeus in Barcelona during 1 year (July 2000–June 2001) were selected.

These patients were diagnosed on the basis of documented allergic reactions following lettuce ingestion. A detailed clinical history of the kind of allergic reactions related to lettuce ingestion was recorded for all subjects, and specific IgE to lettuce was determined by *in vivo* and *in vitro* tests. Open oral challenge tests were performed, except in patients reporting severe life-threatening reactions or recent history of anaphylaxis after lettuce ingestion (29).

Skin prick tests

Skin prick tests (SPT) were performed according to the standard procedure (30) with a panel of inhalant allergens containing the main allergens prevalent in our environment: dust mites, molds, cockroach, cat and dog dander, latex and pollens such as grass, *P. judaica*, *Artemisia vulgaris*, *Plantago lanceolata*, *Chenopodium album*, *Mercurialis annua*, *Olea europea*, *Cupressus arizonica*, *Corylus avellana* and *Platanus acerifolia* (Bial-Aristegui, Bilbao, Spain).

The SPTs with a panel of food allergens with commercially available extracts (Leti, Barcelona, Spain) were also performed, including milk, egg, mixed flours, peach, apple, banana, kiwi, hazelnut, peanut, maize, rice, mustard, celery, green bean, peas,

mixed meat, mixed fish, shrimp, and *Anisakis simplex* and lettuce. We also performed SPT with the prick–prick technique (31) with fresh lettuce leaf and also with implicated foods.

Histamine dihydrochloride 10 mg/ml and phosphate-buffered saline (PBS) solution served as positive and negative controls, respectively. The SPT was considered positive if the wheal area was 3 mm greater than the negative control, 15 min after testing.

Determination of specific IgE

Serum-specific IgE was determined by CAP system-fluorescence enzyme immunoassay method (CAP-FEIA), (Pharmacia Diagnostics, Uppsala, Sweden) and/or by EAST, which was performed with the Allergopharma Spez. IgE ELISA (Allergopharma, Reinbek, Germany), according to the manufacturer's instructions, carrying out double determinations.

Protein extracts and allergens

Protein extracts were prepared from fresh green lettuce and peach by a low temperature acetone powder method as described for apple (32). Freeze-dried plane tree pollen extract was from Bial-Aristegui (Bilbao, Spain). Timothy grass pollen extracts were donated by Dr A. Petersen (Borstel Research Center, Borstel, Germany). Other freeze-dried pollen extracts were purchased from Allergopharma. Recombinant cherry nsLTP Pru av 3 was produced and purified as described (10). Recombinant major birch pollen allergen Bet v 1 was purchased from Biomay (Vienna, Austria). Recombinant cherry profilin Pru av 4 was prepared as described (33).

SDS-PAGE and immunoblotting

Lettuce extract (60 µg protein/cm) was separated under nonreducing conditions by Tricine-SDS-PAGE according to Schägger and V. Jagow using a Bio-Rad (Munich, Germany) Mini Protean cell. 26. The 16% T, 2.6% C separating gel was overlaid by a 4% T, 2.6% C stacking gel. Proteins were transferred onto a 0.2 µm nitrocellulose membrane (Schleicher und Schüll, Dassel, Germany) by tank blotting (Hoefer Pharmacia Biotech Inc., San Francisco, CA, USA) for 45 min. Transfer was controlled by reversible staining of membranes with Ponceau S (Sigma Diagnostics, St Louis, MO, USA). For the detection of allergen-specific IgE antibodies sera were diluted 1 : 5 and incubated with the blot strips overnight. After washing, bound IgE antibodies were detected with alkaline phosphatase-conjugated monoclonal mouse anti-human IgE (1 : 750; PharMingen, San Diego, CA, USA). The binding patterns were visualized with CSPD chemiluminescent Kit (Applied Biosystems, Bedford, MA, USA). For N-terminal sequencing, lettuce extract was transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad), and the protein pattern was stained with Coomassie Brilliant Blue.

N-terminal microsequencing

N-terminal amino acid sequencing was performed with a Procise 492 protein sequencer in pulsed liquid mode (Applied Biosystems, Weiterstadt, Germany).

Immunoblotting inhibition

Immunoblotting inhibition experiments were conducted to identify cross-reactivity between lettuce and several pollens of the Mediter-

anean area such as mugwort, timothy, *Parietaria*, and *Platanus* pollens. Other studies with peach extract, birch pollen and its major allergen rBet v 1, and with two recombinant proteins from cherry rPru av 3 (nsLTP) and rPru av 4 (profilin) were performed in order to identify the lettuce allergens.

Lettuce immunoblotting experiments were carried out with serum of three selected patients. Two of them (patients 5 and 8) were selected because they were only sensitive to a 9 kDa protein from the lettuce extract. The other serum tested (patients 13) was negative to this low molecular weight protein, but displayed IgE reactivity to many proteins with molecular weight above 20 kDa.

Immunoblot inhibition experiments were performed by means of pre-incubating each patient serum sample (120 µl) with 150 µg of lettuce extract protein (as positive control), 150 µg of skim milk protein (as negative control), 150 µg of mugwort, timothy, *Parietaria*, birch, *Platanus* pollen and peach and with 15 µg of rPru av 3 (cherry LTP), rPru av 4 (cherry profilin) and rBet v 1, respectively. Then, the samples were incubated with blot strips of lettuce extract. To detect bound IgE antibodies, the immunodetection was completed according to the standard procedure.

Results

Patients

Fourteen patients, three males and 11 females, aged from 22 to 43 years (mean age, 30.8 years), hypersensitive to lettuce were diagnosed. Seven patients reported anaphylaxis, four, pruritus of the oral cavity, two, urticaria-angioedema and one gastric disorders after lettuce ingestion (Table 1).

All subjects were sensitized to *Platanus* pollen and suffered from pollinosis. Several patients were also sensitized to other pollens such as mugwort (50%), *Parietaria* (21%), and grass (14%). All patients referred a history of pollinosis that had appeared prior to adverse reactions to foods. All patients were positive to lettuce both in SPT with commercial extract and with the fresh food. Most of the patients were sensitized to other plant-

derived foods, and several of the foods causing a positive SPT were not tolerated by the patients, mainly hazelnut (57%) and peach (43%) (Table 2). The highest frequency of clinically insignificant positive STP results was observed with maize (64%).

Immunoblotting reveals a 9 kDa major allergen in lettuce

Ten of the fourteen patients (71%) showed specific IgE binding to a low molecular weight protein of 9 kDa (Fig. 1, lanes 1, 3, 5–12). Notably, all the patients who suffered from anaphylaxis were sensitized to this protein, except two who did not show any IgE reactivity to lettuce extract immunoblot.

The IgE binding protein bands of 25 kDa and higher were detected by sera from two patients (Fig. 1, lanes 3 and 13), with one also showing IgE to the 9 kDa band. One serum (lane 6) had a very strong reactivity to multiple lettuce proteins including the 9 kDa band. Three patients did not show IgE binding to any protein (lanes 2, 4, 14), although two of them had positive specific serum IgE to lettuce (Table 1).

The 9 kDa lettuce allergen is an nsLTP and shows partial cross-reactivity to pollen

Immunoblotting inhibition and N-terminal amino acid sequencing were performed to prove if the 9 kDa band represented an nsLTP. In two patients with IgE binding to the 9 kDa protein (Fig. 2A, lanes 8–21) IgE binding to this protein band was completely inhibited by the cherry LTP rPru av 3 (Fig. 2A, lanes 9, 16) (10), but not by cherry profilin rPru av 4 (33) or by rBet v 1. After blotting onto PVDF and Coomassie staining, an intensive 9 kDa band was excised and subjected to microsequencing, resulting in the sequence: A I S X G Q V T A N L A. The

Table 1. Summary of data of patients with lettuce allergy

Patient no.	Age (years)	Food symptoms	Other sensitizations		IgE lettuce KU/l	IgE Platanus KU/l	9 kDa band	25 kDa band	HMW bands
			Aeroallergens	Foods					
1	32	A	P	h,m,pe	2.4	ND	+		
2	28	A		h, m	<0.35	25.8			
3	33	A	g,mu,cy,o,p,ch	h,pe,a,gb,ps,r,m	2.1	8.14	+	+	+
4	38	OP		h,gb	0.4	>100			
5	29	OP	Mu	h,pe,m	6.8	>100	+++		
6	24	OP	Mi	pe,m,ps	3.6	>100	+		
7	23	A	mi,mu	h,m,r,b	5.1	1.66	++		
8	32	A	Mu	h,r,m	13.8	13.32	+++		
9	22	OP	mu,g	h,pe	1.1	89.08	+		
10	28	A	Mu	pe,gb,h,m	13	4.42	+++		
11	35	U	P		<0.35	24.1	++		
12	43	AE	Mu	pe,m,h	5.3	>100	++		
13	32	GD	Mi	h,sh	13	3.69		+	+
14	32	A	mi,mu	pe	0.9	28.4			

A, anaphylaxis; OP, oral pruritus; U, urticaria; AE, angioedema; GD, gastric disorders; p, parietaria; g, grass; mu, mugwort; cy, cypress; o, olea; ch, chenopodium; mi, mites; m, maize; pe, peach; h, hazelnut; a, apple; gb, green bean; ps, peas; r, rice; b, banana; sh, shrimp; ND, not done; HMW, high molecular bands.

Table 2. Clinical symptoms among patients with food sensitizations

Patient	Peach	Apple	Banana	Hazelnuts	Maize	Green bean	Peas	Rice
1	OP			OP	T			
2				A	T			
3	OP	OP		OP	T	T	T	T
4				AE		T		
5	AE			AE	T			
6	OP				T		OP	
7			AE	AE	T			U
8				A	T			T
9	OP			OP				
10	OP			OP	T	T		
11								
12	T			T	T			
13				OP				
14	OP							

A, anaphylaxis; OP, oral pruritus; U, urticaria; AE, angioedema; T, no symptoms.

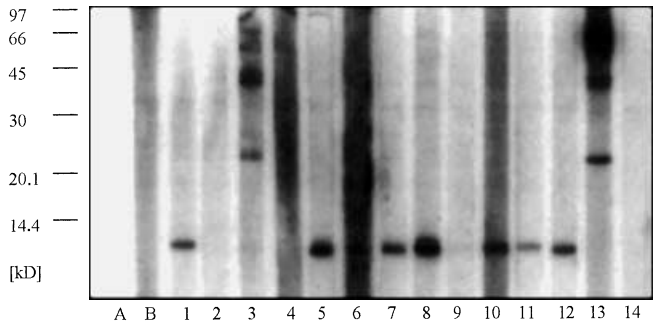


Figure 1. IgE immunoblotting of lettuce extract. Lane A: buffer control; lane B: nonallergic control; lanes 1–14: lettuce-allergic patients.

sequence was submitted to a Fasta3 database search (<http://www2.ebi.ac.uk/cgi-bin/newfasta3.pl?>). The results indicated a high degree of sequence identity to many nsLTP from plants. The highest degree of identity (ID) and similarity (SI) to a known food allergen was found with the apple LTP Mal d 3 (SWISS-PROT Acc.no. Q9M5X7) (ID: 67%, SI: 92%). A clear relationship was also found with allergenic LTP from cherry (Pru av 3, GenBank acc.-no.: AF221501) (ID: 58%, SI: 92%), maize (Zea m 14, Swissprot acc.-no.: P19656) (ID: 58%, SI: 83%), peach (Pru p 3, Swissprot acc.-no.: P81402) (ID: 50%, SI: 75%), and hazelnut (GenBank acc.-no.: AF329829) (ID: 25%, SI: 75%).

In further inhibition experiments, *Platanus* pollen extract caused a clear partial inhibition of IgE antibody binding to this band (Fig. 2A, lanes 14, 21). Pre-incubation of serum with peach extract, known to contain high amounts of LTP (34), completely depleted the 9 kDa band in the two selected sera (Fig. 2B, lanes 14, 21). Weak partial inhibition was obtained with mugwort and parietaria extract, but not with extracts from birch pollen and timothy grass. By contrast, the IgE antibodies to lettuce proteins of higher molecular weight in patient 13

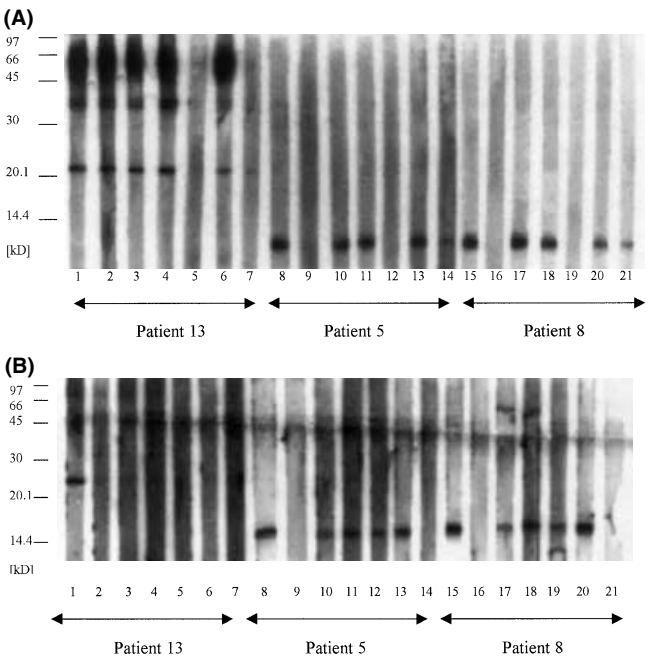


Figure 2. Inhibition of IgE binding to lettuce extract using sera of three selected patients. Lanes 1–7: patient 13. Lanes 8–14: patient 5. Lanes 15–21: patient 8. (A) Inhibitors: lane 1: patient 13 no inhibitor. Lane 2: rPru av 3. Lane 3: rPru av 4. Lane 4: rBet v 1. Lane 5: lettuce extract. Lane 6: skim milk protein. Lane 7: *Platanus* pollen extract. Lane 8: patient 5, no inhibitor. Lane 9: rPru av 3. Lane 10: rPru av 4. Lane 11: rBet v 1. Lane 12: lettuce extract. Lane 13: skim milk protein. Lane 14: *Platanus* pollen extract. Lane 15: patient 8, no inhibitor. Lane 16: rPru av 3. Lane 17: rPru av 4. Lane 18: rBet v 1. Lane 19: lettuce extract. Lane 20: skim milk protein. Lane 21: *Platanus* pollen extract. (B) Inhibitors: lane 1: patient 13, no inhibitor. Lane 2: lettuce extract. Lane 3: mugwort pollen extract. Lane 4: timothy pollen extract. Lane 5: parietaria pollen extract. Lane 6: birch pollen extract. Lane 7: peach extract. Lane 8: Patient 5, no inhibitor. Lane 9: lettuce extract. Lane 10: mugwort pollen extract. Lane 11: timothy pollen extract. Lane 12: parietaria pollen extract. Lane 13: birch pollen extract. Lane 14: peach extract. Lane 15: Patient 8, no inhibitor. Lane 16: lettuce extract. Lane 17: mugwort pollen extract. Lane 18: timothy pollen extract. Lane 19: parietaria pollen extract. Lane 20: birch pollen extract. Lane 21: peach extract.

appeared to be highly cross-reactive to pollen: complete inhibition was obtained with all pollen extracts and peach extract, but not with rPru av 3, rPru av 4 and rBet v 1 (Figs 2A and B, lanes 1–7).

Discussion

Hypersensitivity to lettuce is considered rare, and very few and isolated cases have been reported so far, most frequently corresponding to contact dermatitis. Surprisingly, we identified 14 patients who suffered from allergic reactions after lettuce ingestion within 1 year, and 50% of

the subjects experienced anaphylactic reactions to this food. Lettuce allergy and lettuce anaphylaxis have been rarely reported so far. Probably, lettuce is a food which has been considered too healthy and safe so far (as carrots, apples, etc...). This fact might have made patients and clinicians to overlook it as a potential cause of allergic reactions. New studies about food allergy and food allergens, mainly plant-derived ones, are currently reported. Moreover, some reactions diagnosed in the past as 'idiopathic anaphylaxis' might have been caused by hidden or unknown food allergens as lettuce.

Immunoblotting revealed a 9 kDa protein as the major allergen of lettuce. As IgE binding to this band was strongly inhibited by pure recombinant cherry LTP Pru av 3 and N-terminal amino acid sequencing showed a high degree of sequence identity to many nsLTPs from plants including the apple allergen Mal d 3 (6, 10) and the major peach allergen Pru p 3 (34), the allergen was identified as a member of the nsLTP allergen family. Moreover, the results of our immunoblot studies suggest nsLTP as the cause of systemic reactions, as five of the seven anaphylactic patients had IgE specific for this protein. According to the official allergen nomenclature (<http://www.allergen.org/Pub.htm>), we denominate the major lettuce allergen as Lac s 1.

Several patients reported adverse reactions to other plant-derived foods known to contain homologous nsLTP, mainly to peach and hazelnuts, but symptoms elicited by these foods were not always the same as symptoms elicited by lettuce (Table 2). The immunological relationship of lettuce and peach LTP was confirmed by the strong inhibition of IgE binding to lettuce LTP by peach extract (Fig. 2B). Although the highest degree of sequence identity of the N-terminus of the lettuce-LTP was found with the apple-LTP Mal d 3, only one patient had allergic reactions after apple ingestion. The lack of correlation between sequence identity and clinical cross-reactivity may in part be due to the fact that only a short stretch of the N-terminus of the lettuce LTP was sequenced. It is likely that the overall sequence IDs may be different, and that the compared partial sequences may not include the relevant epitope areas of the LTPs. In contrast to a recent report (35) maize was frequently SPT positive (75%) in our patient panel, but all sensitizations appeared to be clinically irrelevant. In summary, these observations confirm the well-known discrepancy between serum IgE and skin test reactivity on the one hand and clinical reactivity on the other (5, 33, 36, 37).

Double-blind placebo-controlled food challenge (DBPCFC) would be the appropriate tool to determine clinical relevance. As 50% of our patients had episodes of anaphylactic reactions to lettuce, DBPCFC were not performed in this study. However, an open oral challenge fresh lettuce homogenized in water confirmed clinical reactivity in the patients with milder reactions.

Previous cases of systemic reactions after lettuce ingestion have been reported (17, 24–28). In these studies,

the IgE binding components of lettuce extracts were investigated, and all the IgE binding protein bands found had molecular weights ranging from 14 to 100 kDa, but no 9 kDa proteins have been described as lettuce allergens so far (17, 24, 27, 28). This may be due to the fact that all of the previous reports were only isolated case reports. IgE binding to multiple bands ranging from 14 to 100 kDa was also observed in two participants of our study (patients 3 and 13). Moreover, it may be possible that IgE reactivity in the low molecular weight range has been overlooked in previous studies as a result of methodological problems, for example the separation range of electrophoresis, or the fact that the IgE reactivity of LTP is lowered under reducing conditions. During optimization of the blotting method in this study, we observed a three- to five-fold increase in LTP signal intensity under nonreducing conditions (data not shown).

Sera from three patients were negative in the lettuce immunoblot, but two of them showed IgE specific for lettuce in the CAP. Again, the denaturing conditions of the SDS-PAGE may have destroyed conformational epitopes of lettuce proteins recognized by IgE from these patients.

Lipid transfer proteins constitute a plant family widely distributed in the plant kingdom not only in fruits but also in pollens. They are known as acting in plant defense against bacterial and fungal infections (38). Because of their stable structure and high resistance to pepsin digestion, they have been held responsible for more severe allergic reactions, and it is a common belief that sensitization to LTP in food occurs by the oral route (8, 39, 40). In contrast to other pan-allergens, LTPs are considered to be involved in food-allergic reactions without associated pollinosis (6, 39, 41).

In our study, the LTP has been demonstrated to be a major allergen in patients suffering from allergic reactions to lettuce. Also, a strong association with *Platanus* pollen allergy was found.

Several studies have been performed in order to elucidate the cross-reactivity between foods and pollen LTP from different sources (8, 9, 14). Díaz-Perales et al. reported on partial cross-reactivity between Rosaceae LTPs (peach, apple, apricot) and those from different plant sources (mugwort, chestnut) due to common epitopes shared by LTPs from these foods and pollens (9). Other studies demonstrated cross-reactivity between LTPs from Rosaceae and non-Rosaceae foods. Regarding pollens they confirmed cross-reactivity to mugwort pollen whereas grass pollen and birch pollen did not show cross-reactivity with Rosaceae foods (5, 9). Also, the *Parietaria* major allergen has been found to show homology to LTP (14).

In our study, we analyzed for possible cross-reactivity with lettuce LTP, extracts from different relevant pollens of the Mediterranean area, such as *Parietaria*, mugwort, and timothy extract, and others such as birch, ragweed, and *Platanus* pollen extracts. On the one hand, we found

a clear partial cross-reactivity with *Platanus* pollen extract. On the other hand, a clearly higher cross-reactivity was observed with LTPs from plant-derived food such as rPru av 3 (10) and peach extract (34) (Fig. 2A and B), suggesting again that LTP of plant-derived food cause a direct sensitization which occurs through the gastrointestinal route (6, 39, 35, 42). Nevertheless, the IgE cross-reactivity between lettuce-LTP and *Platanus* pollen may indicate the presence of homologous pollen LTP. Silver stained SDS-PAGE of both lettuce and plane pollen extract showed that this 9 kDa band is clearly visible in lettuce, but can hardly be recognized in plane pollen extract (data not shown). This fact might be due to a lower LTP concentration in the plane pollen compared with the lettuce extract, which may, in part, account for

the weaker inhibition by the pollen extract. Considering the strong clinical association between *Platanus* pollinosis and lettuce allergy it is tempting to speculate that a cross-reactive priming by *Platanus* pollen LTP might be involved in the induction of a Th2 response to lettuce LTP.

To our knowledge this is the first report of the identification of an allergenic LTP in lettuce. Although allergy against lettuce has been rarely described so far, it is a plant-derived food that has to be considered as responsible for severe reactions after its ingestion. Studies in patients with *Platanus* pollinosis from other regions of Europe may be useful to prove the relevance of the observed association between sensitization to *Platanus* pollen and LTP in food.

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