New insights into the sensitization to nonspecific lipid transfer proteins from pollen and food: New role of allergen Ole e 7

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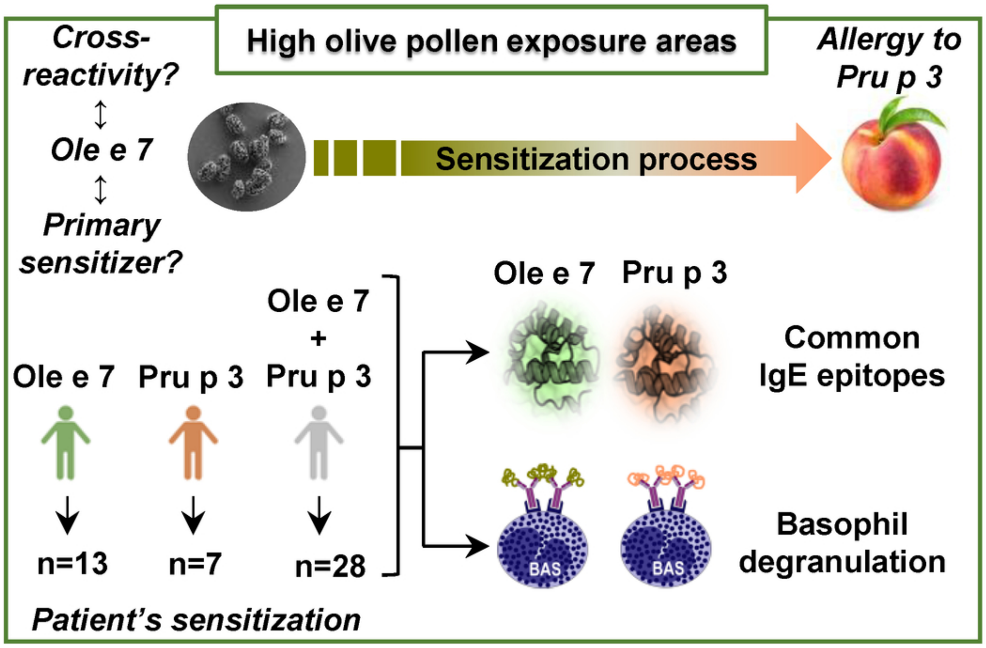
Allergy. 2020 Apr;75(4):798-807.

https://doi.org/10.1111/all.14086



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New insights into the sensitization to non-related nsLTPs from pollen and food: new role of the allergen Ole e 7

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1. Ole e 7 cross-reacts with LTPs from pollen and food, specifically with peach and pear.

2. Common IgG and IgE epitopes were identified between Ole e 7 and Pru p 3 despite their low amino-acid sequence identity.

3. Ole e 7 could act as primary sensitizer in regions with high olive-pollen exposure, leading to Pru p 3 sensitization.

New insights into the sensitization to non-related nsLTPs from pollen and food: new role of the allergen Ole e 7.

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Abstract Word count: 247
Text Word count: 3865

Abstract

Background: Ole e 7 is a non-specific lipid transfer protein (nsLTP) from olive pollen, one of the main allergenic pollens worldwide. This allergenic nsLTP is responsible for severe symptoms in regions with high olive pollen exposure, where many Ole e 7-sensitized patients exhibit a co-sensitization to the peach nsLTP, Pru p 3. However, there is no evidence of cross-reactivity which explains this observed co-sensitization. Therefore, the purpose of this study was to explore the relationship between Ole e 7 and Pru p 3.

Methods: A total of 48 patients sensitized to Ole e 7 and/or Pru p 3 were included in the study. Specific IgE serum levels were measured by ImmunoCAP 250 and ELISA. Inhibition assays were performed to determine the existence of cross-reactivity between both nsLTPs. Allergic response was analyzed *ex vivo* (Basophil Activation Test) and *in vitro* (RBL-2H3 mast cell model).

Results: Common IgG and IgE epitopes were identified between both allergens. IgE-binding inhibition was detected in Ole e 7-monosensitized patients using rPru p 3 as inhibitor, reaching inhibition values of 25 and 100%. *Ex vivo* and *in vitro* assays revealed a response against rPru p 3 in four (31%) Ole e 7-monosensitized patients.

Conclusions: Our results suggest that Ole e 7 could play a new role as primary sensitizer in regions with high olive pollen exposure, leading to the peach nsLTP sensitization. This co-sensitization process would occur because of the cross-reactivity between Ole e 7 and Pru p 3 observed in some allergic patients.

Short title

New insights into non-related nsLTPs sensitization

Keywords

Cross-reactivity, Non-specific Lipid Transfer Protein, Olive pollinosis, Peach allergy, Primary sensitization.

Abbreviations

BAT- Basophil Activation Test
ELISA- Enzyme-Linked ImmunoSorbent Assay
nsLTP- Non-specific Lipid Transfer Protein
SPT- Skin Prick Test
SEM- Standard Error of the Mean
WB- Western Blotting

Introduction

The huge development of proteomics and molecular biology techniques in the last years has boosted the identification of specific proteins responsible for the allergic responses with the aim to improve the diagnosis and immunotherapy protocols of the pathology. The disease exacerbation is dependent on the severity of allergen-specific symptoms or whether allergy is caused by a genuine sensitization (primary or species-specific) or by cross-reactivity (1). Cross-reactivity is due to the existence of IgEs, which recognize the same epitope in allergens from different biological sources (2). These reactions have been widely described among many allergenic protein families, including pollen and food allergens (3-5).

Non-specific Lipid Transfer Proteins (nsLTPs) comprise a protein family of 7-9 kDa, widely distributed throughout the plant kingdom (6). Notably, several members of the nsLTP family are considered main allergens in the Mediterranean area. Among them, Pru p 3, the major allergen from peach, is one of the most frequently recognized fruit allergens and plays a relevant role in cross-reactivity, even with pollen LTPs (7-10). Additionally, Pru p 3 behaves as the primary sensitizer in the development of food allergy to *Rosaceae* species in regions where the presence of birch is rare and the involvement of Bet v 1-like allergens is infrequent, such as in southern Europe (3, 11-13). However, it has been described that Pru p 3 could lose its primary sensitizer role in regions where birch is scarce and the presence of other pollens is intense (14, 15).

Olive tree pollinosis is worldwide increasing due to its extensive cultivar. Indeed, it is a main allergenic source in California (US), China, India, Australia and South America beyond the Mediterranean Basin, where this pollen has been extensively studied (16). Fourteen olive pollen allergens have been identified so far (16-18). Among them, the nsLTP Ole e 7 is a major allergen associated to severe clinical symptoms in regions with high levels of olive pollen counts such as in Andalusia (Spain) (19, 20). In this area, some allergic patients to Ole e 7 show clinical symptoms to *Rosaceae* fruits, being the co-sensitization to Pru p 3 frequently observed.

Despite the 31% of amino acid sequence identity reported between Ole e 7 and Pru p 3 (21), a co-sensitization to these two LTPs has been previously described in other Spanish regions (22). Therefore, an immunological relationship between Ole e 7 and Pru p 3 may not be excluded, with Ole e 7 as the primary sensitizer in the development of peach allergy, especially in areas with high exposure to olive pollen. However, the underlying causes of this co-sensitization have not yet been elucidated.

In this context, the aim of the present work was to explore the role of Ole e 7 in the sensitization process to peach by means of Pru p 3, in those regions with olive pollen counts over 5000 grains/m^3, to get further insights into the underlying causes of a possible co-sensitization. To this end, we performed *in vitro* analysis of the IgE response to both allergens by ELISA and WB, followed by *ex vivo* and *in vitro* cellular response analyses in basophils and the RBL-2H3 mast cell line model.

We reported for the first-time evidences of cross-reactivity between Ole e 7 and Pru p 3, which had not yet been examined. Moreover, we identified four Ole e 7-monosensitized patients who developed a cellular response against Pru p 3, despite their unique sensitization to Ole e 7 by ImmunoCAP 250 and SPT. Our results suggest that Ole e 7 could behave as a primary sensitizer in regions with high olive pollen exposure, leading to a secondary sensitization to Pru p 3 with or without symptoms.

Materials and Methods

See the materials and methods section in this article's supporting information online for full details about protein extracts, allergens and antibodies, IgG-binding analysis, specific IgE binding to Ole e 7 and Pru p 3, cell-based allergic mediator release assays, and ELISA and WB inhibition assays.

Patients

The study was approved by the Ethical Committees of the Reina Sofía University Hospital, Córdoba, Spain (ref. 3033), and the Complutense University and Instituto de Salud Carlos III (CEI P49). Written informed consent was obtained from all patients. All samples were anonymously handled.

A total of 48 patients recruited at the Immunology and Allergy Department of the Reina Sofía University Hospital (Córdoba, Spain) with a confirmed history of allergy to olive pollen with sensitization to Ole e 7 and/or sensitization to LTP from peach (Pru p 3) were included in the study. Clinical evaluation included examination of patient history, SPT and determination of specific IgE (sIgE). The SPT was performed according to the European guidelines (23), using commercial extracts from *Olea europaea* pollen and peach (ALK-Abelló, Madrid, Spain). A positive SPT response was considered when the diameter of the wheal was 3 mm greater than that induced by the negative control. sIgE to Ole e 7 and Pru p 3 were measured by ImmunoCAP 250 (Phadia, Uppsala, Sweden) according to the manufacturer recommendations.

Patients were stratified according to their sensitization status by ImmunoCAP 250 (Supporting Table 1). In addition, patients were also analyzed by SPT to *O. europaea* and Pru p 3. Accordingly, three groups of study were defined:

I. Ole e 7-monosensitized patients (n=13) had sIgE to Ole e 7 >0.35 kU/L and sIgE to Pru p 3 <0.35 kU/L. In addition, a positive SPT response to *O. europaea* pollen and a negative SPT response to LTP-peach were observed in all patients.

II. Pru p 3-monosensitized patients (n=7) had sIgE to Pru p 3 >0.35 kU/L and sIgE to Ole e 7 <0.35 kU/L. In addition, a positive SPT response to Pru p 3 was observed in 71.43% of patients and a negative SPT to *O. europaea* pollen in all patients.

III. Ole e 7 and Pru p 3-bisensitized patients (n=28) had a sIgE level >0.35 kU/L to both allergens. In addition, 60.71% of these patients showed a positive SPT response to Pru p 3 and 92.86% to *O. europaea* pollen.

Patients sensitized to Ole e 7 exhibited rhinoconjunctivitis and bronchial asthma due to the exposure to olive pollen. Patients sensitized to Pru p 3 had a clinical history of allergic reactions following the ingestion of *Rosaceae* fruits. Symptoms of food allergy included oral allergy syndrome (OAS), urticaria/angioedema, gastrointestinal symptoms or anaphylaxis. None of the patients had been treated with immunotherapy for the last three years before the inclusion in the study or was being treated with active immunotherapy.

Results

Ole e 7 displays IgE-cross-reactivity with nsLTPs from food-derived extracts

Previous studies have described cross-reactions between several LTPs from different biological sources. Nevertheless, the role of Ole e 7 in these processes has not been clarified due to the low amount of natural Ole e 7 allergen obtained after its purification. The successful expression of Ole e 7, whose primary amino acid sequence was obtained by proteomics and retains the structural, allergenic and antigenic properties of the natural allergen (17), has enabled a deeper analysis of the cross-reactivity of Ole e 7 with other LTPs. First, we surveyed different pollen and food-derived extracts by an ELISA inhibition test to determine whether any LTP could cross-react with Ole e 7. To this end, six pollen and ten food-derived extracts were used as inhibitors of the IgE-binding to Ole e 7 (Fig 1A, B). Regarding pollen extracts, *O. europaea* used as control of the assay was almost able to completely inhibit the IgE-binding to Ole e 7. In addition, other members from the Oleaceae family showed significant inhibition values: 80% *L. vulgare* from Ligustrum genus*,* 56% *S. vulgaris* from Syringa genus and 65% *F. excelsio*r from Fraxinus genus (Fig 1A). Moreover, the highest significant inhibition with food-derived extracts was observed with peach (*P. persica)* and pear (*P. communis)* extracts as inhibitors, with significant inhibition values of 67 and 52% respectively (Fig 1B).

These results suggest the existence of cross-reactions between Ole e 7 and LTPs from pollen and food-derived extracts.

Ole e 7 and Pru p 3 share common IgG epitopes

We next focused the study on the potential LTP pollen-food cross-reactivity between Ole e 7 and Pru p 3 because of their clinical relevance, even though they possess low amino acid sequence identity (Supporting Fig 1).

Prior to establish structural relationships analyzing the IgG recognition of both LTPs, the quality of the recombinant allergens was assessed by Coomassie blue staining and mass spectrometry (Fig 2A, Supporting Fig 2). Then, the IgG-binding to each allergen from each specific polyclonal antiserum to rOle e 7 or rPru p 3 was analyzed by WB and ELISA (Fig 2B-D).

Although Ole e 7 was also barely recognized by the Pru p 3 polyclonal antiserum by WB, Ole e 7 and Pru p 3 were recognized by both polyclonal antisera (Fig 2B). Then, the IgG-binding to the native proteins was analyzed by ELISA (Fig 2C, D). In agreement with the previous results obtained by WB, Pru p 3 polyclonal antiserum was able to recognize Ole e 7. In contrast, although the specific Ole e 7 polyclonal antiserum slightly recognized Pru p 3, these results show the presence of common IgG epitopes in both LTPs, which could also cause their cross-reactivity at IgE level.

To address this question, our next aim was to study the existence of common IgE epitopes, and further analyzed their potential IgE cross-reactivity by cellular assays.

Some patients may recognize equivalent IgE epitopes from olive pollen and peach nsLTPs.

Inhibition assays by ELISA and WB were performed using individual sera from two Ole e 7-monosensitized and four bisensitized patients, whose sIgE levels were measured by ELISA and ImmunoCAP (Supporting Fig 3, Supporting Fig 4 and Supporting Table 1). Both proteins were used on the solid phase and as inhibitors (Fig 3, Supporting Fig 4).

Interestingly, the bisensitized patient 44 used as control of IgE reactivity to both LTPs showed IgE-binding inhibition by ELISA as well as WB. By ELISA, a complete inhibition of the IgE-binding to rOle e 7 was obtained using both rOle e 7 and rPru p 3 as inhibitors. In contrast, inhibition to rPru p 3 was barely observed using rOle e 7 as inhibitor (Fig 3A, B). By WB, a complete inhibition to the IgE-binding to rOle e 7 was achieved using rOle e 7 and rPru p 3 as inhibitors, and a 25% IgE-binding inhibition to rPru p 3 was reached using rOle e 7 as inhibitor (Fig 3C, D). In addition, three other bisensitized patients were analyzed by ELISA (Supporting Fig 4). For patients 1 and 27, Ole e 7 or Pru p 3 as inhibitors were almost able to abrogate the IgE binding to each protein. On the other hand, similar results with that of patient 44 were also obtained for patient 21. These results indicate, apart from the heterogeneous IgE reactivity of patients, that some IgE epitopes of Pru p 3 seem to be absent in Ole e 7, especially in the native conditions of the protein. However, we cannot discard that these epitopes could not be highly recognized by sIgEs from patients, being the affinity for those epitopes decisive to identify the epitopes involved in cross-reactions. Even so, although further experiments would be performed to determine the relevance of the different affinity of the IgE, the inhibition of the IgE-binding observed in the experiments suggest the existence of common conformational and linear epitopes supporting the cross-reactivity between both allergens.

Surprisingly, the two Ole e 7-monosensitized patients analyzed by WB showed an inhibition of the IgE binding to rOle e 7 of 25 and 100%, using rPru p 3 as inhibitor (Fig 3E). No IgE binding was detected to rPru p 3 strips by any patient. Furthermore, we explored the differences on the IgE recognition to those epitopes under denaturing and non-denaturing conditions. A reduction of the signal of about 15% was detected under non-denaturing conditions (Fig 3F). The lack of conformational epitopes of the protein in the presence of β-ME (denaturing conditions) suggests, besides the existence of common conformational epitopes observed by ELISA with bisensitized patients, the existence of common linear IgE epitopes for both allergens.

Analysis of the release of cell mediators reveals that Ole e 7 could trigger the allergic response to the nsLTP from peach in Ole e 7-monosensitized patients.

Next, we proceeded to study this reactivity at IgE level in the forty-eight patients sensitized to Ole e 7 and/or Pru p 3 according to ImmunoCAP 250 (Supporting Figure 3 and Supporting Table 1) by means of *ex vivo* cellular assays by BAT (Figure 4, Supporting Figure 5) and by *in vitro* analysis using the RBL-2H3 mastocyte cell model (Figure 5).

Regarding the Ole e 7 response by BAT (Fig 4A), we observed that the percentage of degranulated basophils was significantly higher in Ole e 7-monosensitized and bisensitized patients than in Pru p 3-monosensitized patients (p=0.001 and p<0.001, respectively), whereas no significant differences were found between Ole e 7-monosensitized and bisensitized patients (p=0.864). Interestingly, when considering the Pru p 3 response (Fig 4B), no significant differences between the three analyzed groups were found. Surprisingly, we identified four Ole e 7-monosensitized patients whose basophils degranulated after the incubation with rPru p 3. Thus, while basophils from Pru p 3-monosensitized patients were not able to degranulate in the presence of Ole e 7, basophils from 4 out of 13 Ole e 7-monosensitized patients were also able to degranulate in the presence of Pru p 3.

To confirm the specificity of the observed response, BAT was performed at 0.1 µg/mL, 1 µg/mL and 10 µg/mL of rOle e 7 and rPru p 3 in the four Ole e 7-monosensitized patients who responded to Pru p 3, and two random selected bisensitized patients. Clinical characteristics of these patients are summarized in Table 1. Basophils from the four Ole e 7-monosensitized patients (patient 9, 12, 16 and 36) degranulated similarly at the three concentrations of rOle e 7. However, when rPru p 3 was used to promote degranulation, the response was only achieved at the highest concentration. These results show that apart from rOle e 7, rPru p 3 can induce basophil degranulation from Ole e 7-monosensitized patients. Basophils from the bisensitized patient 37 behaved like the four-rOle e 7-monosensitized patients (Fig 4C, D), in line with the serum sIgE levels obtained by ELISA, in which IgE was detected only with rOle e 7 (Supporting Fig 3). Regarding bisensitized patient 38, its degranulation percentage was similar at the three different concentrations either with rOle e 7 or rPru p 3 as stimulus (Fig 4C, D).

Lastly, to further confirm these results, these patients sera were analyzed using the RBL-2H3 mastocyte cell model, which allows the measurement of the release of ß-hexosaminidase. Four different concentrations of allergen were used (Fig 5). Interestingly, despite their unique sensitization to Ole e 7, all Ole e 7-monosensitized patients tested were able to react with rPru p 3. Regarding bisensitized patients 37 and 38, although a similar reaction to rOle e 7 was observed at the highest concentration of allergen, the response of patient 38 was mainly produced with rPru p 3 as stimulus. In this patient, these results may suggest the role of Pru p 3 as primary sensitizer.

Collectively, the herein presented results show the existence of common conformational and linear IgE-binding epitopes in some allergic olive pollen patients that could explain the pollen-food cross-reactivity observed in clinics between Ole e 7 and Pru p 3.

Discussion

Cross-reactivity between pollen and food LTPs have been extensively demonstrated, although the identification of the primary inducer in these patients is still unclear (3, 7, 24, 25). A common feature of LTP-allergic patients is their multiple plant-food sensitizations, which is known as LTP syndrome, a high relevant hypersensitivity disorder in the Mediterranean basin due to its association with severe symptoms, such as anaphylaxis (26, 27). nsLTPs from artemisia (Art v 3) and plane tree (Pla a 3) pollens are highly implicated in this syndrome since they cross-react with LTPs from different foods (28). Although this association has not been described for Ole e 7 or Par j 1, which show low amino acid sequence similarity with nsLTPs from foods (26), their role in the LTP syndrome is still unclear. On the other hand, the sensitization to LTPs seems to be usually related to peach-specific IgE levels (26). In southern Europe, the main allergen from peach -Pru p 3- is considered a primary sensitizer for the development of allergy to other pollen and plant-food LTPs (10, 29). However, in regions with high pollen exposure (i.e. mugwort or ragweed), Pru p 3 may lose its role as primary sensitizer (14, 15).

We used in the study sera from patients from the south of Spain where olive tree is widely cultivated and its pollen is the main cause of allergy in these populations. Co-sensitization to Ole e 7 and Pru p 3 has been reported in several patients from this area (22, 30), but no cross-reactivities between these two nsLTP have been described to date. Indeed, preliminary results of our group using the natural Ole e 7 allergen isolated from pollen pointed out to an absence of cross-reactivity (16, 31). In this sense, it is necessary to take into account that the natural allergen isolated from pollen is a heterogeneous mixture of multiple isoforms obtained in a very low concentration and that the experiments were carried out using pools of several sera from allergic patients to Ole e 7. In this context, the main objective of the present study was to clarify whether Ole e 7 is involved in cross-reactivity and which is its implication in the sensitization process to other nsLTPs.

Here, we have demonstrated that Ole e 7 cross-reacts, not only with LTPs from pollens, but also with LTPs from pear and peach (Pyr c 3 and Pru p 3, respectively) in spite of the low sequence identity reported for them (Supporting Figure 1). The obtained results are in agreement with a previous study which established a clinical association between severe food allergic clinical symptoms and sensitization to Ole e 7 (32). Nevertheless, other studies do not support that correlation (21, 33). The observed differences may be attributed to the multifactorial and heterogeneous character of this disorder and the molecules involved in it. Hence, the differences between the studied populations may be explained by the demographic characteristics of each area linked to different environmental factors (34-36). Furthermore, we have here confirmed the antigenic and allergenic cross-relations between Ole e 7 and Pru p 3. Common IgG epitopes were observed although the signal detected for Ole e 7 by the specific polyclonal antiserum against Pru p 3 was much lower than that for Pru p 3, which can be directly related to the affinity of the IgG-binding or the number of recognized epitopes. Common IgE epitopes were also identified. In addition, the conformation of the protein seems to be crucial for the IgE-binding, being recognized mainly under reducing conditions as confirmed by IgE inhibition WB assays. However, the elucidation of which IgE epitopes were recognized in both allergens was not feasible because the 3D structure and the epitope map of Ole e 7 are still unknown, in contrast to the deep analysis so far performed for Pru p 3, whose IgE-binding epitopes have been well characterized (37). Further structural and immunological studies of allergens from the LTP protein family are required to determine which are the main epitopes recognized by allergic patients sensitized to different LTPs.

Remarkably, although an absence of recognition was observed by ELISA and WB, we have identified Ole e 7-monosensitized patients whose basophils reacted against rPru p 3, which was further supported using the RBL-H3 mast cell model. This could be due to the fact that cellular assays are more sensitive than ELISA and WB; or alternatively because low affinity epitopes not recognized by specific IgEs by ELISA or WB were able to trigger a cellular response (38, 39). On the other hand, the absence of lipids in rPru p 3 could also partially affect the detection of some sIgE levels from patients. In addition, none of the patients had clinical symptoms to peach or other *Rosaceae* members, as well as some allergens whose co-sensitization had been diagnosed (37). The obtained results make us to raise the possibility that these patients are in an early stage of the sensitization process to *Rosaceae* (Pru p 3), acting Ole e 7 as the primary LTP sensitizer promoted by the high olive pollen exposure in Andalusia, as it was described for Artemisia pollen in other regions (3, 7, 14, 22). A similar hypothesis was proposed to explain the linking between food allergen and grass (40), or birch pollinosis (41). On the other hand, we cannot discard a different sensitization route by airways instead of the gastrointestinal *via* described for food allergens sensitization. Indeed, Pérez-Calderón et al. suggested that occupational allergy to peach arises as a consequence of the inhalation of Pru p 3 present in the leaves of the plant, and not as a result of fruit ingestion (42). In addition, the study performed by Garcia et al. also supports these data. The authors also confirmed that Pru p 3 from peach leaves act as a respiratory allergen, causing occupational rhinoconjunctivitis and asthma (43). Marzban et al. also raised this alternative mechanism for allergy development in their study of the sensitization to birch pollen and Mal d 1 and Mal d 3 allergens (44, 45). The influence of other cofactors should also be considered because they are frequently involved in the development of the allergic response and in the clinical expression of allergic symptoms (46). Among these factors, the mucosa and the microbiome could also play an important role. The mucosa immune system regulates the entrance of multiple antigens either pathogenic or non-pathogenic, such as food and pollen allergens. Interestingly, it has been described that in spite of the absence of food allergy, the oral mucosa could be compromised due to a respiratory sensitization (47). A disrupted barrier could be responsible for food allergy development, which support our hypothesis about the sensitization process in some analyzed patients. On the other hand, in food allergy, there have been reported differences in the oral and intestinal microbiome that may increase the susceptibility to develop sensitization to food allergens (48). Therefore, the microbiome of the oral mucosa, a shared entrance place between pollen and food allergens, could influence the co-existence of different allergenic processes. Further studies to clarify the mechanism of the co-sensitization between these two allergens are required for a better diagnosis and treatment.

In conclusion, our study demonstrates the cross-reactivity between the nsLTP from olive pollen and peach, a subject of debate for years. Furthermore, data herein presented provide new insights regarding the sensitization process to fruit nsLTP, particularly to Pru p 3. Our results suggest that Ole e 7 could act as a primary sensitizer in regions with high olive pollen exposure, probably leading the peach sensitization by a new route, the respiratory tract. However, further studies are needed to determine which factors are crucial in the onset of the allergic symptoms to peach.

Author Contributions: Conception and design: C.O-S., A. N., C.M-A., A.J., M.V., R.B. Development of methodology: C.O-S., A.N., S.B., L.V. Perform Research: C.O-S., A. N., S.B., B.R-L., A.J. Analysis and interpretation of data: C.O-S., A. N., S.B., B.R-L., A.J., R.B., C.M., M.V. Writing, review, and/or revision of the manuscript: C.O-S., A. N., A.J., C.M., M.V., R.B. Technical, obtaining and processing of samples, or material support: A.J., L.V., C. M-A., A. D-P., A.J., M.V., R.B.

Acknowledgements

This work was supported by SAF2014-53209-R and SAF2017-86483-R grants from the Ministerio de Economía y Competitividad to R.B. and M.V., and to M.V., respectively, and RIRAAF Network RD12/0013/0015 grant and ARADyAL Network RD16/0006 grant from the Instituto de Salud Carlos III (ISCIII) co-founded by Fondo Europeo de Desarrollo Regional -FEDER- for the Thematic Networks and Co-operative Research Centres. B.R., A.J., A.N, and C.M. acknowledge PI-01119-2016 from the Consejería de Salud (Junta de Andalucía) and the Alergosur Foundation. We also gratefully acknowledge the financial support from the ISCIII for the PI17CIII/00045 grant to R.B. S.B. was a recipient of the Juan de la Cierva programme of the MINECO. We also thank the excellent technical support of Sara Abián.

Supporting Information

Supporting information to this article can be found online.

Conflicts of Interest Statement

The authors declare no conflicts of interest.

Tables

			Olive pollen sensitization			Peach sensitization		
Patient	Sex	Age (Y)	Symptoms (RC/A/RCA)	SPT	IgE Ole e7 (CAP)	Symptoms (No/OAS/U/An)	SPT	IgE Pru p 3 (CAP)
9	F	41	RCA	+	201	No	-	0
12	F	23	RCA	+	42.9	No	-	0
16	F	22	RCA	+	58.9	No	-	0
36	M	48	RCA	+	125	No	-	0
37	F	18	A	+	178	No	-	1.13
38	M	22	A	+	25.8	OAS/U	+	25.8
44	F	44	RCA	+	8.71	OAS/U	+	20.8

Table 1. Clinical characteristic of patients selected to perform BAT analysis. M, male; F, female; RC, rhinitis; A, asthma; RCA, rhinitis and asthma; No, No symptoms; OAS, oral allergy syndrome; U, urticaria; An, anaphylaxis; SPT, skin prick test; IgE, immunoglobulin E.

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Legend to the Figures

FIGURE 1. Identification of non-specific lipid transfer proteins cross-reactive to Ole e 7 from different pollen and food-derived extracts. ELISA inhibitions were performed with 20 or 200 µg of pollen (A) or food (B) protein extracts as inhibitors. A pool of six Ole e 7-positive sera from olive pollen allergic patients sensitized to Ole e 7 was used in the assay. Experiments were performed in duplicate ($p<0.05$).

FIGURE 2. Physico-chemical and immunological analysis of recombinant Ole e 7 and Pru p 3. (A) Analysis of 500 ng of the recombinant allergens by Coomassie Blue staining after SDS-PAGE. (B-D) Analysis of common antigenic features of Ole e 7 and Pru p 3 allergens by WB and ELISA. (B) Immunostaining of 500 ng of rOle e 7 and Pru p 3 using the indicated polyclonal antisera (pAb). BSA nitrocellulose blotted was used as negative control of the assay. Signal was developed for 30 seconds using ECL WesternBright™ QUANTUM (Advansta) reagent for Ole e 7 with anti-Ole e 7 pAb and Pru p 3 with anti-Pru p 3 pAb, and for 3 min for the other determinations. (C-D) Analysis of 100 ng of recombinant allergens coated in ELISA wells with indicated dilutions of Ole e 7- or Pru p 3-pAbs. All the experiments were performed in duplicate.

FIGURE 3. IgE-binding inhibition assays using sera from bisensitized and Ole e 7-monosensitized patients. (A-D) ELISA and WB IgE-binding inhibition assays using sera from a patient bisensitized to Ole e 7 and Pru p 3. (E-F) WB IgE-binding inhibition assays using sera from a patient monosensitized to Ole e 7. ELISA was performed coating 100 ng of recombinant proteins using indicated amounts of inhibitors. WB was performed using 200 ng of Pru p 3 or 500 ng of Ole e 7 blotted in nitrocellulose membranes and 2 µg of the indicated inhibitors under denaturing (C-E) or non-denaturing conditions (F). Inhibition values (%) are indicated.

FIGURE 4. Basophils degranulation (% CD63$^+$) by rOle e 7 and r Pru p 3. (A) 10 µg/mL of rOle e 7, or (B) rPru p 3 in blood samples of the study cohort (n=48). Comparisons between groups of patients of the study cohort (n=48) were performed using U Mann Whitney test. Furthermore, basophil degranulation of the 6 selected patients was analyzed using 0.1 µg/mL, 1 µg/mL and 10 µg/mL of rOle e 7 (C) or rPru p 3 (D). N-Formil-Met-Leu-Phe was used as positive control and phosphate buffer saline as negative control. Mean with standard error of the mean (SEM) bars are displayed.

FIGURE 5. β-hexosaminidase release assay of Hum RBL-2H3 cells. Hum RBL-2H3 cells were tested at four different concentrations (10^{-3} µg/mL, 10^{-2} µg/mL, 10^{-1} µg/mL, 1 µg/mL) of rOle e 7 or r Pru p 3 using serum of indicated patients to sensitize the cells overnight. Patients 9, 12, 16 and 36 were monosensitized to Ole e 7. Patients 37 and 38 showed bisensitization to Ole e 7 and Pru p 3. An absence of β-hexosaminidase release was observed without allergen stimulation (negative control).

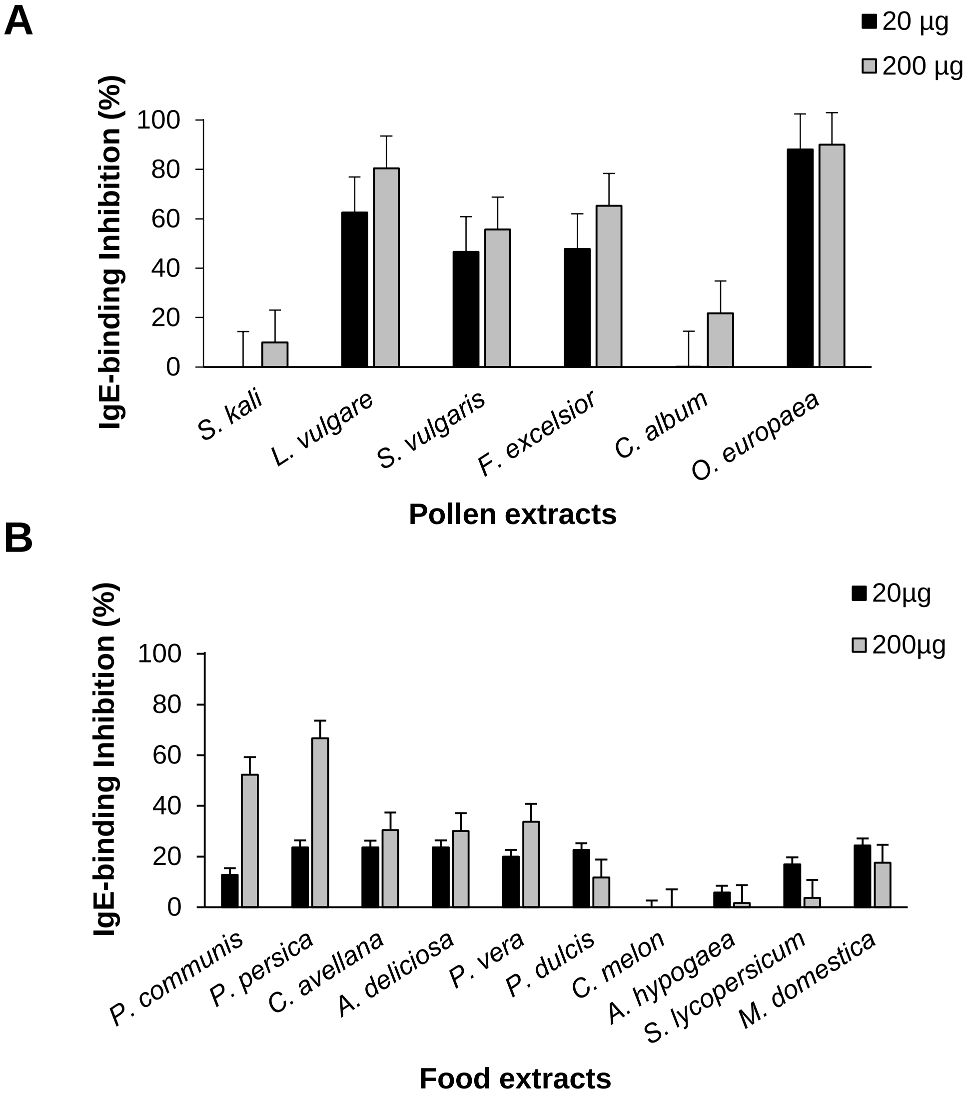
A
■ 20 µg
▨ 200 µg
IgE-binding Inhibition (%)
100
80
60
40
20
0
S. kali
L. vulgare
S. vulgaris
F. excelsior
C. album
O. europaea
Pollen extracts
B
■ 20µg
▨ 200µg
IgE-binding Inhibition (%)
100
80
60
40
20
0
P. communis
P. persica
C. avellana
A. deliciosa
P. vera
P. dulcis
C. melon
A. hypogaea
S. lycopersicum
M. domestica
Food extracts

Figure 1

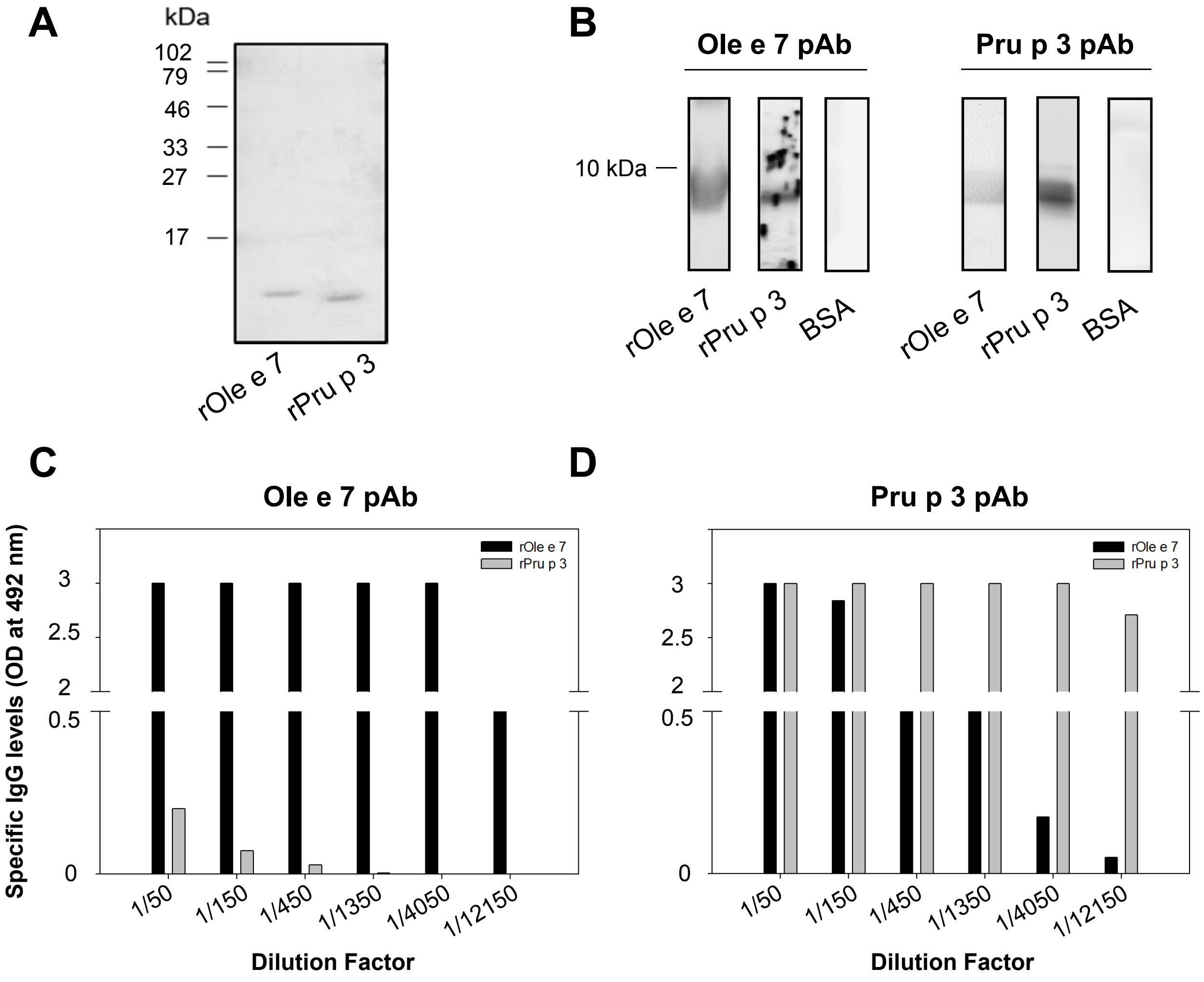
A
kDa
102
79
46
33
27
17
rOle e 7
rPru p 3
B
Ole e 7 pAb
Pru p 3 pAb
10 kDa
rOle e 7
rPru p 3
BSA
rOle e 7
rPru p 3
BSA
C
Ole e 7 pAb
D
Pru p 3 pAb
Specific IgG levels (OD at 492 nm)
rOle e 7
rPru p 3
3
2.5
2
0.5
0
1/50
1/150
1/450
1/1350
1/4050
1/12150
Dilution Factor

Figure 2

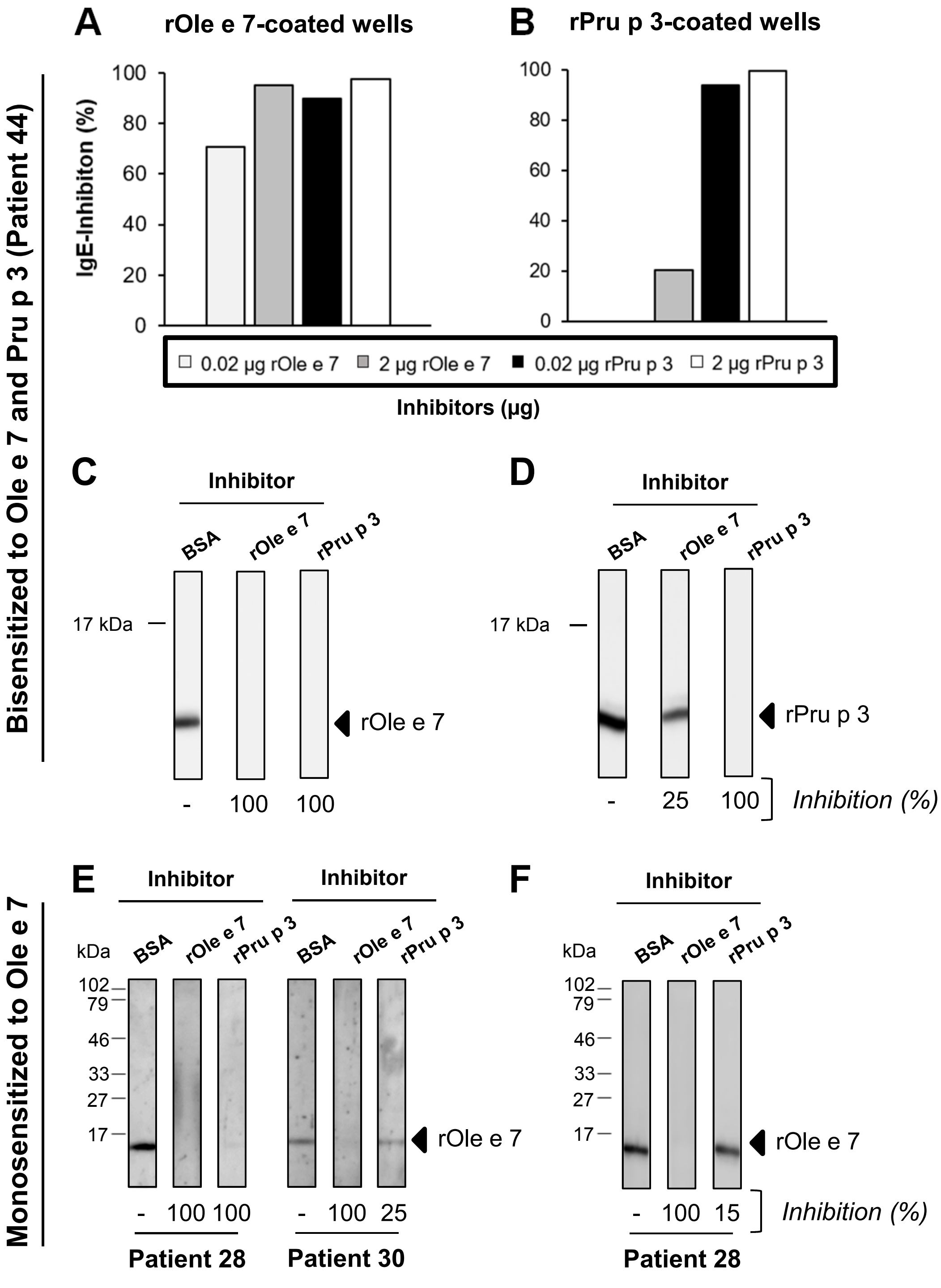
A
rOle e 7-coated wells
B
rPru p 3-coated wells
IgE-Inhibiton (%)
0
20
40
60
80
100
□ 0.02 µg rOle e 7
■ 2 µg rOle e 7
■ 0.02 µg rPru p 3
□ 2 µg rPru p 3
Inhibitors (µg)
Bisensitized to Ole e 7 and Pru p 3 (Patient 44)
C
D
Inhibitor
BSA
rOle e 7
rPru p 3
17 kDa
rOle e 7
rPru p 3
- 100 100
- 25 100
Inhibition (%)
Monosensitized to Ole e 7
E
F
kDa
102
79
46
33
27
17
- 100 100
- 100 25
- 100 15
Patient 28
Patient 30
Patient 28

Figure 3

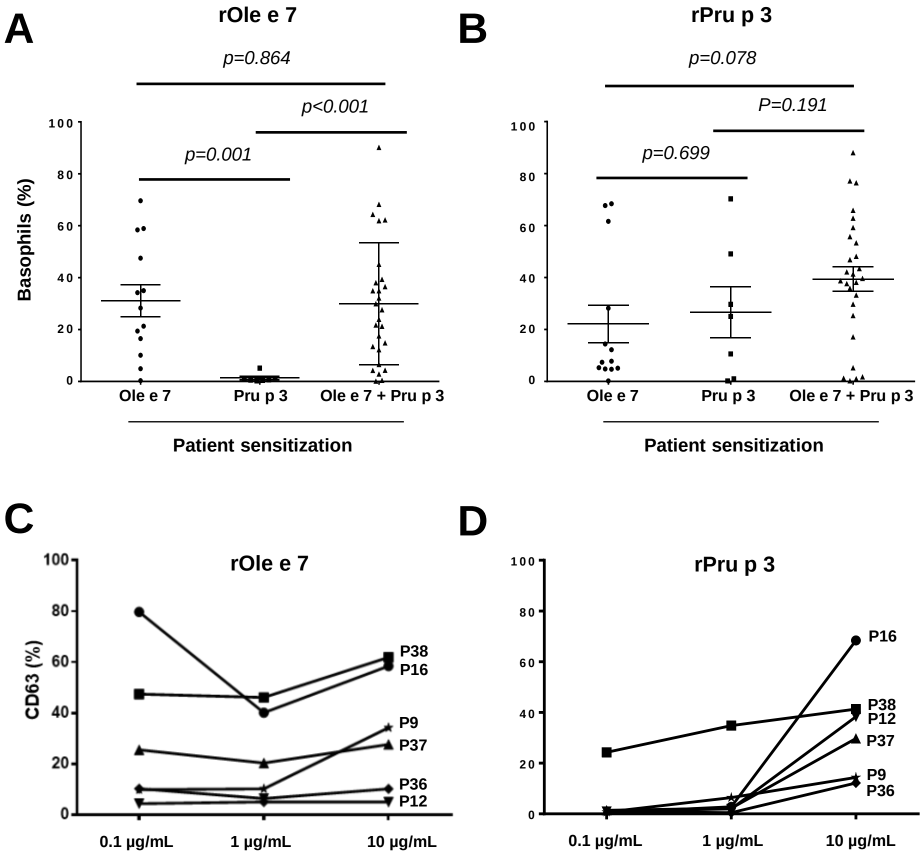
A
rOle e 7
p=0.864
p<0.001
p=0.001
Basophils (%)
100
80
60
40
20
0
Ole e 7
Pru p 3
Ole e 7 + Pru p 3
Patient sensitization
B
rPru p 3
p=0.078
P=0.191
p=0.699
100
80
60
40
20
0
Ole e 7
Pru p 3
Ole e 7 + Pru p 3
Patient sensitization
C
rOle e 7
CD63 (%)
100
80
60
40
20
0
P38
P16
P9
P37
P36
P12
0.1 µg/mL
1 µg/mL
10 µg/mL
D
rPru p 3
100
80
60
40
20
0
P16
P38
P12
P37
P9
P36
0.1 µg/mL
1 µg/mL
10 µg/mL

Figure 4

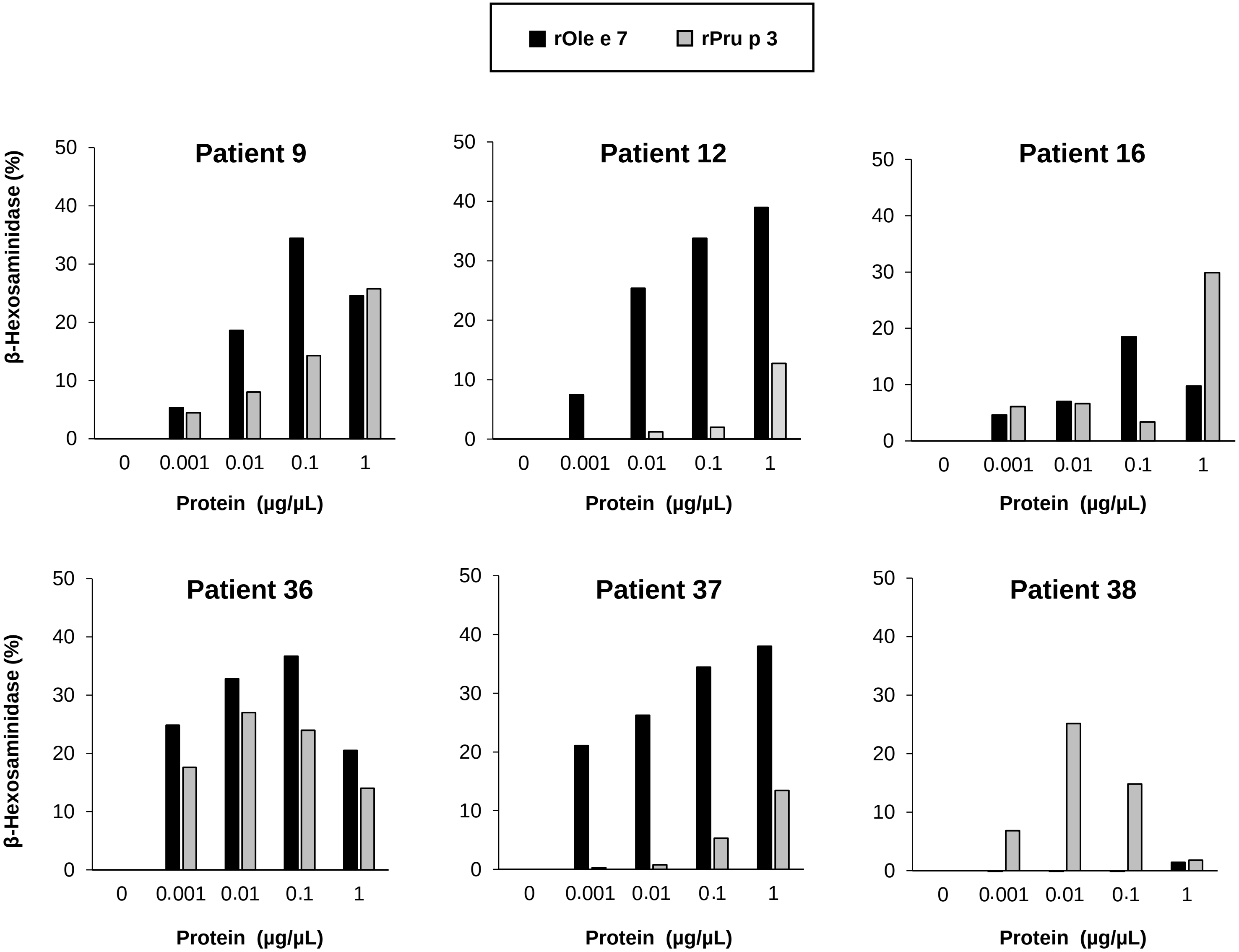
■ rOle e 7
▨ rPru p 3
Patient 9
Patient 12
Patient 16
Patient 36
Patient 37
Patient 38
β-Hexosaminidase (%)
0
10
20
30
40
50
0
0.001
0.01
0.1
1
Protein (µg/µL)

Figure 5

Supporting Information

New insights into the sensitization to non-related nsLTPs from pollen and food

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Materials and Methods

Protein extracts, allergens and antibodies

Olive pollen was obtained from ALK-Abelló (Madrid, Spain). Pollens from other biological sources were obtained from Allergon (1). Pollen protein extracts were prepared by saline extraction. To prepare food-derived extracts, plant foods were crushed under liquid nitrogen to obtain fine flour. Proteins were extracted by homogenization in 0.2 M ammonium bicarbonate buffer, pH 8.0, containing 1 mM PMSF and stirred for one hour at 4ºC. Delipidation was performed using cold acetone. Protein extract concentration was determined according to Lowry et al. (2).

Ole e 7 and Pru p 3 expression and purification were performed according to established protocols (3, 4). Purity of both allergens was confirmed by mass spectrometry on an Autoflex III MALDI-TOF-TOF instrument (Bruker Daltonics, Bremen, Germany), respectively (Supporting Figure 2).

Ole e 7 and Pru p 3 rabbit polyclonal antisera (pAb) were previously obtained accomplishing the ethics guidelines of the Complutense University of Madrid and Fundación Jiménez Díaz, respectively (3-6). Horseradish peroxidase-labeled goat polyclonal antibody against rabbit IgG was purchased from BioRad Laboratories Inc (Richmond, CA, USA). Horseradish peroxidase-labeled mouse anti-human IgE was purchased from Southern Biotech.

IgG-binding analysis

The IgG binding of Ole e 7- and Pru p 3-specific polyclonal antisera to Ole e 7 and Pru p 3 allergens was analyzed by ELISA and WB. Indirect ELISA was performed in duplicate in 96-well plates (Corning™) coated with 0.1 µg of the indicated proteins. A titration of the specific polyclonal antisera to Ole e 7 and Pru p 3 was performed with concentrations ranging from 10^{-2} to 10^{-6} µg/µL. Alternatively, a goat anti-rabbit IgG horseradish peroxidase-labeled antibody (1:3000) or a goat anti-mouse IgG horseradish peroxidase-labeled antibody (1:2500) was used to determine the IgG recognition to each allergen. For WB analysis, proteins were transferred to nitrocellulose membrane after SDS-PAGE under reducing conditions (2-mercaptoethanol 5%). Immunodetection was achieved by incubating with specific polyclonal antisera to Ole e 7 and Pru p 3 (1:10000, 1:1000, respectively) and an anti-rabbit IgG horseradish peroxidase-labeled polyclonal antibody (1:3000). Chemiluminescent signal was developed using ECL-Western blotting reagent (Amersham Bioscience) or WesternBright™ QUANTUM (Advansta) reagents.

Specific IgE binding to Ole e 7 and Pru p 3

Specific IgE levels were determined by ImmunoCAP 250 (Thermo Scientific, Uppsala, Sweden) and indirect ELISA. Values >0.35 kU/L and OD at 492 nm >0.1 by ImmunoCAP 250 and ELISA, respectively, were considered positive. Indirect ELISA was performed in duplicate in 96-well plates (Corning™) coated with 0.1 µg of recombinant protein per well, according to previously optimized protocols (1, 3, 7). Binding of human IgE was detected by a horseradish peroxidase-labeled mouse anti-human IgE Fc (1:1000) (Southern Biotech). Peroxidase reaction was detected by using 50 µL per well of 0.63 mg/mL o-Phenylenediamine in 0.1 M sodium citrate 4% methanol containing 1.6 µL/mL 30% H_2O_2. The reaction was stopped with 50 µL 3N H_2SO_4. Signal was measured at 492 nm in an iMark microplate absorbance reader (Bio-Rad).

Cell-based allergic mediator release assays

Evaluation of specific cell mediators of allergic response against Ole e 7 and Pru p 3 was determined by Basophil Activation Test (BAT) and RBL-2H3 activation cells. BAT was performed using rOle e 7 and rPru p 3 allergens obtained as described above. Briefly, 100 µL of heparin anticoagulated total blood from each allergic patient were incubated with Basophil Stimulation Buffer (ref. 339664, BD Biosciences, CA, USA) for 10 minutes at 37 ºC. Then, 100 µL of phosphate buffer saline (PBS) was added as negative control and 50 µL of N-Formyl-Met-Leu-Phe (f-MLP) at 2 µM concentration as positive control. Either rOle e 7 or rPru p 3 allergens were also added at 0.1 µg/mL, 1 µg/mL and 10 µg/mL in separate tubes. All tubes were incubated for 30 minutes at 37 ºC. Basophil degranulation was stopped by transferring sample tubes to an ice bath for 5 minutes. Cell staining was performed using CD63-FITC/CD123-PE/anti-HLA-DR-PerCP cocktail (ref. 341068, BD FastImmune™, Becton, Dickinson and Company, San Jose, CA, USA). After lysing cells with 2 mL of 1x BD FACS™ lysing solution and washing twice with PBS, stained cells were acquired in a BD FacsCanto II cytometer (Becton Dickinson and Company, San Jose, CA, USA) using BD FacsDiva™ as acquisition and analysis software. The specificity of the response to rOle e 7 and rPru p 3 was ascertained using a cohort of non-allergic donors.

RBL-2H3 cell experiments were developed as previously described (3), with slight modifications. Briefly, cells were sensitized overnight at 37ºC with 5 or 10% of sera from allergic patients. After 12 h, cells were stimulated with either rOle e 7 or rPru p 3 at four different concentrations (10^{-3} µg/mL, 10^{-2} µg/mL, 10^{-1} µg/mL and 1 µg/mL).

Inhibition assays

Inhibition assays were performed by ELISA and WB. For ELISA inhibition tests, 0.1 µg of purified proteins were coated overnight at 4ºC to Corning™ 96 well plates. Samples were coated in duplicates. Individual human sera (diluted 1:10) or an equivolumetric pool of human sera (n=6), previously incubated with 0.2 µg or 2 µg of the specific recombinant protein, or with 20 µg or 200 µg of pollen or food-derived extracts as inhibitors. IgE-binding was detected by a horseradish peroxidase-labeled mouse anti-human IgE Fc (1:1000) (Southern Biotech).

For WB inhibition assays, allergenic proteins were alternatively transferred under denaturing and non-denaturing conditions to analyze the relevance of epitope conformation in IgE-binding. Hence, under denaturing conditions, proteins showed mostly linear epitopes, while under non-denaturing conditions binding to conformational epitopes may be determined. Immunodetection on membranes was achieved by incubating with individual human sera (1:10), previously incubated with 2 µg of inhibitor, and a horseradish peroxidase-labeled mouse anti-human IgE Fc (1:1000). Chemiluminescent signal was developed using ECL-Western blotting reagent (Amersham Bioscience) or WesternBright™ QUANTUM (Advansta) reagents.

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                   *        20         *        40         *        60         *        80         *
Ole e 7  APSCSTVTAKLTSCVSYLDDKSAKPTSDCCAGVKTVLAQATSKPDKLATCNCLKSALALVGNKVDTGRVSSLPKKCGMSVDLPPLDKNYDCSKVP  95
Pru p 3  -ITCGQVSSALAPCIPYVRGGGAVPPA-CCNGIRNVNNLARTTPDRQAACNCLKQLSASV-PGVNPNNAAALPGKCGVHIPYK-ISASTNCATVK  91
Pyr c 3  AITCSQVSANLAPCINYVRSGGAVPPA-CCNGIKTINGLAKTTPDRQAACNCLKNLAGSV-SGVNPGNAESLPGKCGVNVPYK-ISTSTNCATVK  92
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	Identity (%)			***Similarity (%)***		
Ole e 7	100	31	30	100	51	46
	Ole e 7	Pru p 3	Pyr c 3	Ole e 7	Pru p 3	Pyr c 3

Supporting Fig 1. Comparison of the amino acid sequence of Ole e 7 with Pru p 3 and Pyr c 3. Identity and Similarity percentage values among indicated allergenic nsLTPs are shown. Dialign was used to align the amino acid sequences of the proteins. Genedoc was used for visualization and calculation of Identity and Similarity percentages.

Supporting Information

New insights into the sensitization to non-related nsLTPs from pollen and food

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Materials and Methods

Protein extracts, allergens and antibodies

Olive pollen was obtained from ALK-Abelló (Madrid, Spain). Pollens from other biological sources were obtained from Allergon (1). Pollen protein extracts were prepared by saline extraction. To prepare food-derived extracts, plant foods were crushed under liquid nitrogen to obtain fine flour. Proteins were extracted by homogenization in 0.2 M ammonium bicarbonate buffer, pH 8.0, containing 1 mM PMSF and stirred for one hour at 4ºC. Delipidation was performed using cold acetone. Protein extract concentration was determined according to Lowry et al. (2).

Ole e 7 and Pru p 3 expression and purification were performed according to established protocols (3, 4). Purity of both allergens was confirmed by mass spectrometry on an Autoflex III MALDI-TOF-TOF instrument (Bruker Daltonics, Bremen, Germany), respectively (Supporting Figure 2).

Ole e 7 and Pru p 3 rabbit polyclonal antisera (pAb) were previously obtained accomplishing the ethics guidelines of the Complutense University of Madrid and Fundación Jiménez Díaz, respectively (3-6). Horseradish peroxidase-labeled goat polyclonal antibody against rabbit IgG was purchased from BioRad Laboratories Inc (Richmond, CA, USA). Horseradish peroxidase-labeled mouse anti-human IgE was purchased from Southern Biotech.

IgG-binding analysis

The IgG binding of Ole e 7- and Pru p 3-specific polyclonal antisera to Ole e 7 and Pru p 3 allergens was analyzed by ELISA and WB. Indirect ELISA was performed in duplicate in 96-well plates (Corning™) coated with 0.1 µg of the indicated proteins. A titration of the specific polyclonal antisera to Ole e 7 and Pru p 3 was performed with concentrations ranging from 10^{-2} to 10^{-6} µg/µL. Alternatively, a goat anti-rabbit IgG horseradish peroxidase-labeled antibody (1:3000) or a goat anti-mouse IgG horseradish peroxidase-labeled antibody (1:2500) was used to determine the IgG recognition to each allergen. For WB analysis, proteins were transferred to nitrocellulose membrane after SDS-PAGE under reducing conditions (2-mercaptoethanol 5%). Immunodetection was achieved by incubating with specific polyclonal antisera to Ole e 7 and Pru p 3 (1:10000, 1:1000, respectively) and an anti-rabbit IgG horseradish peroxidase-labeled polyclonal antibody (1:3000). Chemiluminescent signal was developed using ECL-Western blotting reagent (Amersham Bioscience) or WesternBright™ QUANTUM (Advansta) reagents.

Specific IgE binding to Ole e 7 and Pru p 3

Specific IgE levels were determined by ImmunoCAP 250 (Thermo Scientific, Uppsala, Sweden) and indirect ELISA. Values >0.35 kU/L and OD at 492 nm >0.1 by ImmunoCAP 250 and ELISA, respectively, were considered positive. Indirect ELISA was performed in duplicate in 96-well plates (Corning™) coated with 0.1 µg of recombinant protein per well, according to previously optimized protocols (1, 3, 7). Binding of human IgE was detected by a horseradish peroxidase-labeled mouse anti-human IgE Fc (1:1000) (Southern Biotech). Peroxidase reaction was detected by using 50 µL per well of 0.63 mg/mL o-Phenylenediamine in 0.1 M sodium citrate 4% methanol containing 1.6 µL/mL 30% H_2O_2. The reaction was stopped with 50 µL 3N H_2SO_4. Signal was measured at 492 nm in an iMark microplate absorbance reader (Bio-Rad).

Cell-based allergic mediator release assays

Evaluation of specific cell mediators of allergic response against Ole e 7 and Pru p 3 was determined by Basophil Activation Test (BAT) and RBL-2H3 activation cells. BAT was performed using rOle e 7 and rPru p 3 allergens obtained as described above. Briefly, 100 µL of heparin anticoagulated total blood from each allergic patient were incubated with Basophil Stimulation Buffer (ref. 339664, BD Biosciences, CA, USA) for 10 minutes at 37 ºC. Then, 100 µL of phosphate buffer saline (PBS) was added as negative control and 50 µL of N-Formyl-Met-Leu-Phe (f-MLP) at 2 µM concentration as positive control. Either rOle e 7 or rPru p 3 allergens were also added at 0.1 µg/mL, 1 µg/mL and 10 µg/mL in separate tubes. All tubes were incubated for 30 minutes at 37 ºC. Basophil degranulation was stopped by transferring sample tubes to an ice bath for 5 minutes. Cell staining was performed using CD63-FITC/CD123-PE/anti-HLA-DR-PerCP cocktail (ref. 341068, BD FastImmune™, Becton, Dickinson and Company, San Jose, CA, USA). After lysing cells with 2 mL of 1x BD FACS™ lysing solution and washing twice with PBS, stained cells were acquired in a BD FacsCanto II cytometer (Becton Dickinson and Company, San Jose, CA, USA) using BD FacsDiva™ as acquisition and analysis software. The specificity of the response to rOle e 7 and rPru p 3 was ascertained using a cohort of non-allergic donors.

RBL-2H3 cell experiments were developed as previously described (3), with slight modifications. Briefly, cells were sensitized overnight at 37ºC with 5 or 10% of sera from allergic patients. After 12 h, cells were stimulated with either rOle e 7 or rPru p 3 at four different concentrations (10^{-3} µg/mL, 10^{-2} µg/mL, 10^{-1} µg/mL and 1 µg/mL).

Inhibition assays

Inhibition assays were performed by ELISA and WB. For ELISA inhibition tests, 0.1 µg of purified proteins were coated overnight at 4ºC to Corning™ 96 well plates. Samples were coated in duplicates. Individual human sera (diluted 1:10) or an equivolumetric pool of human sera (n=6), previously incubated with 0.2 µg or 2 µg of the specific recombinant protein, or with 20 µg or 200 µg of pollen or food-derived extracts as inhibitors. IgE-binding was detected by a horseradish peroxidase-labeled mouse anti-human IgE Fc (1:1000) (Southern Biotech).

For WB inhibition assays, allergenic proteins were alternatively transferred under denaturing and non-denaturing conditions to analyze the relevance of epitope conformation in IgE-binding. Hence, under denaturing conditions, proteins showed mostly linear epitopes, while under non-denaturing conditions binding to conformational epitopes may be determined. Immunodetection on membranes was achieved by incubating with individual human sera (1:10), previously incubated with 2 µg of inhibitor, and a horseradish peroxidase-labeled mouse anti-human IgE Fc (1:1000). Chemiluminescent signal was developed using ECL-Western blotting reagent (Amersham Bioscience) or WesternBright™ QUANTUM (Advansta) reagents.

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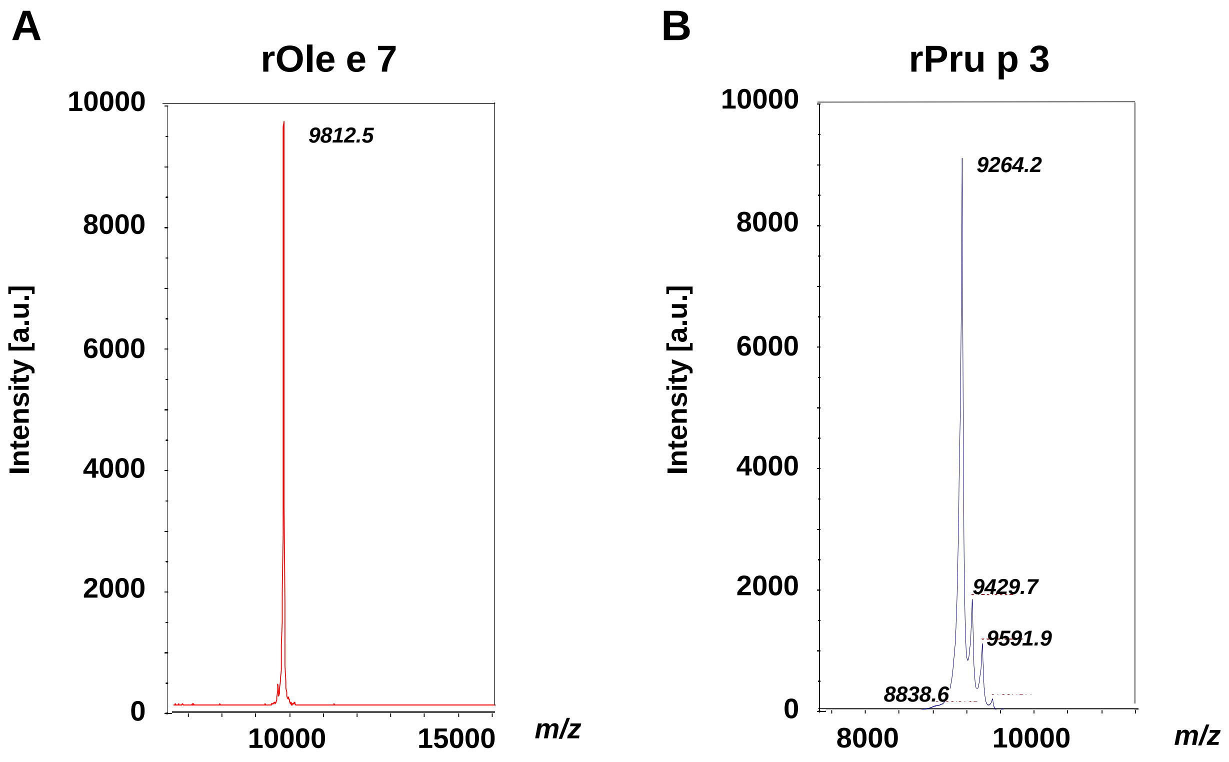
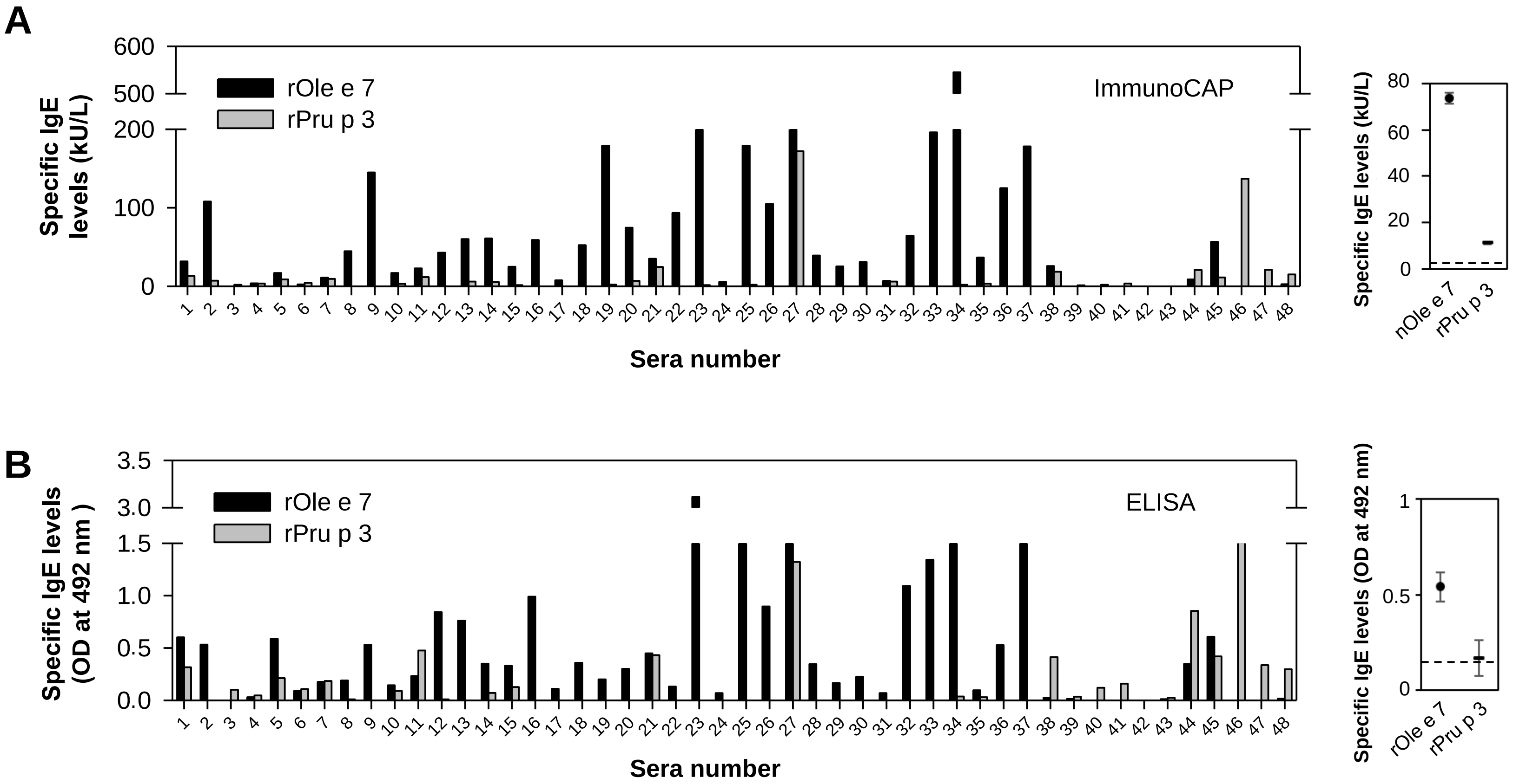
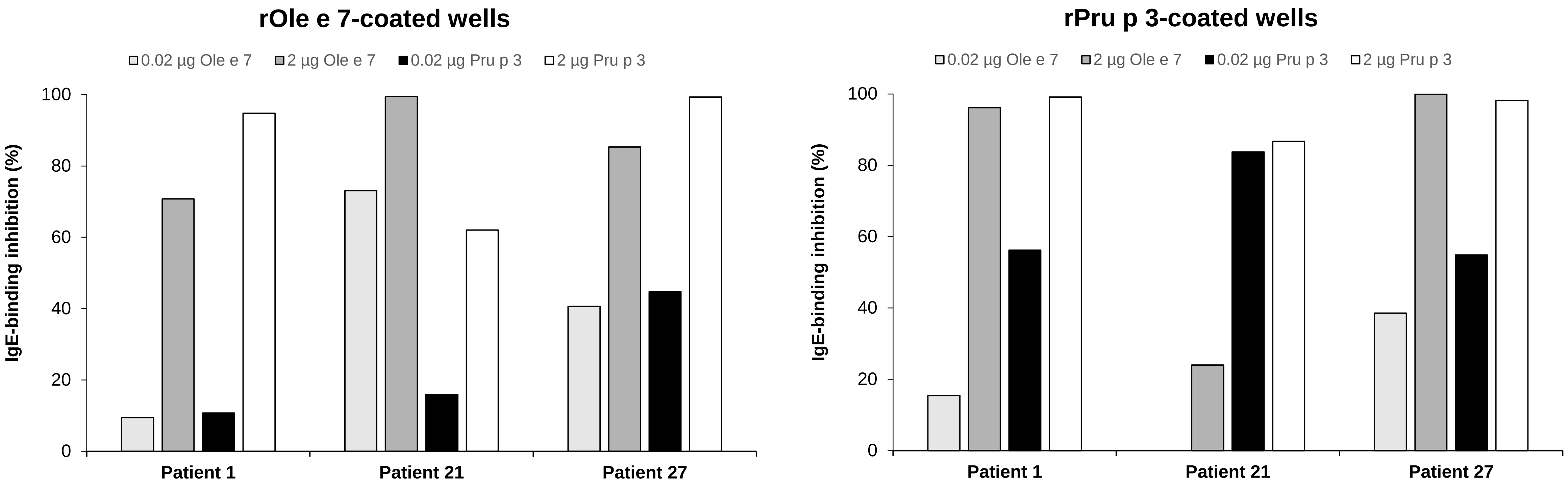


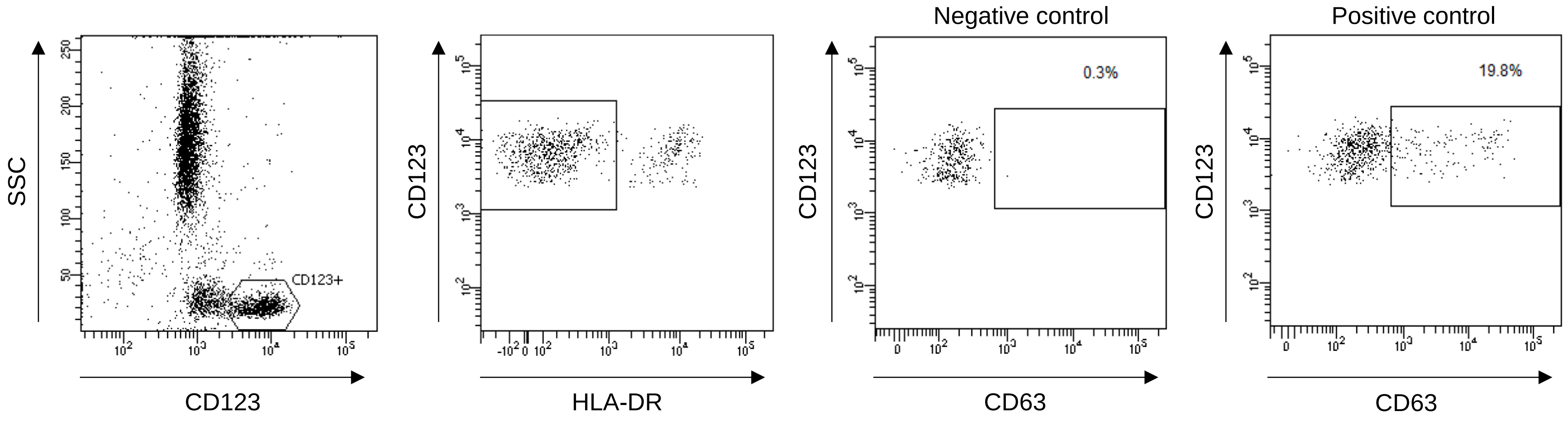
Supporting Fig 2. Quality control of the recombinant proteins of the study after expression and purification. Purity of rOle e 7 and rPru p 3 was confirmed by mass spectrometry (MALDI-TOF-TOF).



Supporting Fig 3. Detection of specific IgE levels from serum of patients to Ole e 7 and Pru p 3 by ImmunoCAP (A) and ELISA (B). Values above 0.1 OD by ELISA and 0.35 kUA/L by ImmunoCAP were considered positive. Right, Mean ± standard error of the mean (SEM) of the IgE binding to Ole e 7 (natural or recombinant protein for ImmunoCAP and ELISA, respectively) and rPru p 3 for all positive serum from Córdoba are also represented.



Supporting Fig 4. IgE-binding inhibition assay by ELISA from bisensitized patients to Ole e 7 and Pru p 3. Inhibition of the IgE-binding to 100 ng of rOle e 7 or rPru p 3 was determined by ELISA in comparison to the IgE-binding signal without inhibitor. Amounts of inhibitor (µg) are also indicated in the figure.



Supporting Fig 5. Gating strategy. SSC, side scatter. Basophils were identified according to the expression of CD123 and HLA-DR markers (CD123+HLA-DR-). Degranulated basophils were identified according to the expression of CD63 marker (CD63+). Results from healthy individual blood cells stimulated with phosphate buffer saline (negative control) and N-Formil-Met-Leu-Phe (positive control) are shown.

	Monosensitized to Ole e 7 (n=13)	**Monosensitized to Pru p 3 (n=7)**	**Bisensitized (n=28)**
Age, mean (SD)	36.7 (10.14)	42.14 (14.49)	31.46 (10.27)
Female, (%)	46.15	85.71	60.71
Habitat			
Rural (%)	46.15	14.29	50
Urban (%)	53.85	85.71	50
Respiratory clinical symptoms			
Rhinitis (%)	15.38	28.57	21.43
Asthma (%)	0	14.29	7.14
Both (%)	84.62	42.86	64.29
Clinical symptoms after Rosaceae consumption			
Cutaneous (%)	0	28.57	14.29
Cutaneous and OAS (%)	0	0	14.29
OAS (%)	0	42.86	14.29
Anaphylaxis (%)	0	28.57	42.86
No symptoms (%)	0	0	7.14
Positive SPT to *Olea europaea* (%)	100	0	92.86
Positive SPT to Pru p 3 (%)	0	71.43	60.71
IgE to Ole e 7 by ImmunoCAP, mean (SD)	62.37 (42.50)	0.11 (0.08)	97.39 (142.11)
IgE to Pru p 3 by ImmunoCAP, mean (SD)	0.08 (0.12)	23.65 (50.51)	12.92 (31.84)
IgE to rOle e 7 by ELISA, mean (SD)	0.49 (0.35)	0.06 (0.13)	0.71 (0.97)
IgE to rPru p 3 by ELISA, mean (SD)	0 (0)	0.22 (0.30)	0.25 (0.45)

Supporting Table 1. Clinical characteristics of all recruited patients to whom Basophil Activation Test (BAT) was performed. OAS, oral allergy syndrome; IgE, immunoglobulin E. IgE values are indicated in kU/L for ImmunoCAP or in DO (arbitrary units).

Supporting Table 1