Pla l 1 and Ole e 1 Pollen Allergens Share Common Epitopes and Similar Ultrastructural Localization

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Abstract

Background: English plantain (*Plantago lanceolata* L.) and olive (*Olea europaea* L.) pollens are important causes of pollinosis in large areas of North America, Australia, and the Mediterranean basin. The major pollen allergens of both plants, Pla l 1 and Ole e 1, share 38.7% of their amino acid sequences.
Objective: To analyze putative cross-reactivity between these 2 proteins.
Methods: Several antibodies and patients' sera were used in immunoblot and immunocytochemistry experiments.
Results: Two anti-Pla l 1antibodies were able to bind to 3 polypeptides from olive pollen protein extracts, which correspond to the 3 glycosylation isoforms of Ole e 1 (18-22 kDa) previously described. Moreover, Pla l 1 protein was found in the cytoplasm of both the vegetative and the generative cells of *P lanceolata* mature pollen. On olive pollen sections, these anti-Pla l 1 antibodies displayed significant labeling in the cytoplasm of the vegetative cell and in both the exine and the material adhering to this outer layer of the pollen wall. In addition, the anti-Ole e 1 antibody 10H1 was found to cross-react with proteins of similar masses (16-20 kDa) to Pla l 1 variants. In *Plantago* pollen sections, the 10H1 antibody recognized proteins located in the cytoplasm of both the vegetative and generative cells. Cross-reaction was confirmed using sera from patients allergic to either plant pollen.
Conclusion: Both allergens share common epitopes, which can be cross-recognized by different antibodies and sera from different patients, although this antigenic similarity seems to have little clinical relevance.

Key words: Cross-reactivity. Localization. Ole e 1. Pla l 1. Pollen.

Resumen

Antecedentes: Los pólenes de llantén (*Plantago lanceolata L.*) y olivo (*Olea europaea L.*) son importantes causas de polinosis en amplias zonas de América del Norte, Australia y el área Mediterránea. Los alérgenos mayoritarios de ambas plantas, denominados Pla l 1 y Ole e 1 respectivamente, comparten un 38.7% de sus secuencias aminoacídicas.
Objetivo: Analizar la posible reactividad cruzada entre dichos alérgenos.
Métodos: Se usaron diversos anticuerpos y sueros de pacientes en experimentos de inmunoblot e inmunocitoquímica.
Resultados: Dos anticuerpos anti-Pla l 1 fueron capaces de reaccionar con tres polipéptidos de los extractos proteicos de polen de olivo, que corresponden a las tres formas de glucosilación de Ole e 1 (18-22 kDa) previamente descritas. Además, la proteína Pla l 1 fue localizada en el citoplasma de las células vegetativa y generativa del polen maduro de *P lanceolata*. Sobre secciones de polen de olivo, dichos anticuerpos anti-Pla l 1 produjeron un marcado significativo tanto en el citoplasma de la célula vegetativa como en la exina y el material adherido a esta capa externa de la pared del polen. Por otra parte, el anticuerpo 10H1 anti-Ole e 1 mostró reacción cruzada con proteínas de pesos moleculares aparentes similares a las variantes de Pla l 1 (16-20 kDa). En secciones de polen de *Plantago*, el anticuerpo 10H1 reconoció proteínas localizadas en el citoplasma de las células vegetativa y generativa. La reacción cruzada fue confirmada usando sueros de pacientes alérgicos al polen de una u otra planta.
Conclusión: Ambos alérgenos comparten epítopos comunes, que pueden ser reconocidos por distintos anticuerpos y sueros de pacientes, aunque la relevancia clínica de esta similitud antigénica parece ser escasa.

Palabras clave: Localización. Ole 1. Pla l 1. Polen. Reactividad cruzada.

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Introduction

Cross-reactivity between allergens plays a major role in triggering the IgE-mediated response [1], and allergy symptoms in sensitized patients often appear after they come into contact with a second allergen. Therefore, the presence of specific IgEs against an allergen does not necessarily mean that the patient has been directly exposed to that allergen. In pollens, these allergenic cross-reactions are common between taxonomically related species [2], although they can also occur between unrelated botanical families [3]. Profilins, which have been defined as panallergens, are responsible for the cross-reactivity detected between many unrelated plant species [4,5]. The major allergen from *Betula verrucosa* (Bev v 1) and some lipid transfer proteins are also responsible for these cross-reactions between fruit and pollen allergens [6,7]. Cross-reactivity is widely accepted to be linked to the presence of common epitopes [1]. In this context, the carbohydrate moieties of many allergens have also been reported to be responsible for cross-reactivity [8].

Olive (*Olea europaea L.*) pollen is one of the main causes of allergy by inhalation in the Mediterranean area [9]. In Spain, this is the second agent for pollinosis after grass pollen and the major allergen, Ole e 1, represents up to 20% of total protein of the pollen grain [10]. This 20-kDa acidic protein consists of a single 145-amino acid polypeptide chain, which exhibits 3 glycosylation variants in addition to microheterogeneity at several positions [11].

The highest incidence of English plantain (*Plantago lanceolata*) pollen in allergy has been reported in Australia [12] and Europe [13], and a high prevalence of this pollen has been described in pollinosis in Spain [14,15]. The major allergen from *Plantago*, Pla l 1, is a hydrophilic glycoprotein with a polypeptide chain of 131 amino acid residues that represents up to 80% of the allergenic capacity of the pollen [16]. At least 4 different variants ranging from 16 kDa to 20 kDa have been described. Since the protein possesses a single N-glycosylation site, other post-translational modifications or sequence polymorphisms may account for Pla l 1 heterogeneity [17]. A sequence similarity search disclosed a significant degree of homology with Ole e 1 [18]. The amino acid identity of both allergens is 38.7%, and this percentage rises to 68.2% when conservative changes are considered. Therefore, both allergens are good candidates for testing cross-reactivity. In this study, we adopted biochemical, immunological, and cytochemical approaches using monoclonal antibodies and a panel of sera from patients.

Methods

Plant Materials

Mature pollen grains were collected from *Olea europaea* L. trees (cv. "Picual") in Granada (Spain). For this purpose, olive inflorescences containing dehiscent anthers were vigorously shaken in large paper bags. Pollen grains were separated from debris by sieving the material through an appropriate mesh.

Dehiscent pollen grains and anthers containing mature pollen grains were collected from wild populations of *Plantago lanceolata* L. in Granada, Spain. For each flower sampled, 1 anther was squashed in a solution of acetic carmine and observed under a light microscope for staging. The remaining anthers were immediately frozen and stored in liquid nitrogen or directly processed for electrophoresis or microscopy.

Protein Extraction and Western Blot Analysis

Protein extracts were prepared from *P lanceolata* and *O europaea* "Picual" pollens by stirring 1 g of material in 10 mL of a solution containing 50 mM Tris-HCl (pH 7.5), 300 mM NaCl, 1 mM EDTA, 1 mM EGTA, 2% v/v Triton X-100, 5 mM ascorbic acid, 100 mM DTT, and 2 mM PMSF for 8 h at 4°C. Samples were then centrifuged at 12 000*g* for 10 min at 4°C, and the resulting supernatants were stored at –20ºC until use. The protein concentration was assayed by the Bradford method [19] using the Bio-Rad reagent (Bio-Rad, Hercules, California, USA).

For immunoblotting, 25 μg of total protein of each sample was separated by sodium dodecyl sulfate polycrylamide gel electrophoresis (SDS-PAGE) in 12% gels as described by Schägger et al [20]. Proteins were then transferred onto polyvinylidene difluoride (PVDF) membranes at 100 V for 1.5 h using a Mini Trans-Blot Electrophoretic device (Bio-Rad). For immunodetection, the resulting membranes were probed for 12 h with the Pla l-specific 2A10, 6G10 [21], or the Ole e 1-specific 10H1 [22] monoclonal antibodies diluted 1/50 in blocking reagent (3% w/v bovine serum albumin and 0.3% v/v Tween 20 in Tris-buffered saline). A rabbit antimouse IgG-alkaline phosphatase-conjugated antibody (Promega Corporation, Madison, Wisconsin, USA) was used as the secondary antibody (dilution 1/2000). The color was developed by using 4-nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP, Amresco, Solon, Ohio, USA) as substrate.

Sera were obtained from individuals who were allergic to either plantain or olive pollen, as demonstrated by clinical history, positive skin reaction, and presence of serum-specific IgE (radioallergosorbent test class 2-4), or acquired from PlasmaLab International (Everett, Washington, USA). When probing immunoblots with individual sera from sensitized patients, the membranes were thoroughly washed and incubated with an antihuman IgE antibody conjugated to peroxidase. The signal was detected with a 3,3' diamino-benzidine tetrahydrochloride solution (Sigma-Aldrich Chemicals, St. Louis, Missouri, USA) or by chemiluminescence as described by Calabozo et al [16].

Immunocytochemistry

Dehiscent olive pollen and excised mature anthers of plantain were fixed and processed as described by Castro et al [10]. For light microscopy studies, non-specific binding sites were blocked by incubation in 5% w/v BSA and 0.1% v/v Tween 20 in PBS for 30 min. After blocking, Pla l 1 allergen was immunolocalized by treatment with the 2A10 monoclonal antibody (1 mg/mL) for 12 h. Sections were washed 3 times in PBS for 20 min each, and incubated with a goat anti mouse IgG alkaline phosphate-conjugated secondary antibody (Sigma-Aldrich Chemicals, St. Louis, Missouri, USA) diluted 1/1000 in PBS for 1 h. The slides were then washed 3 × 20

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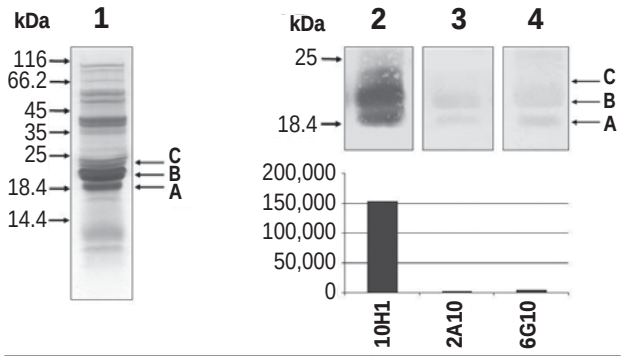

Figure 1. Sodium dodecyl sulfate polycrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis of Ole e 1 allergen in crude extracts of pollen collected from "Picual" olive trees. Lane 1: Coomassie blue staining of total protein. Lane 2: immunoblot as above, probed with the anti-Ole e 1 antibody (10H1), and relative densitometric data corresponding to the Ole e 1 band. Lanes 3-4: immunoblots as above probed with 2 monoclonal antibodies (2A10 and 6G10, respectively) raised against the major allergen of *P lanceolata*, Pla l. The graphs represent the corresponding densitometric data relative to the cross-reacting bands.

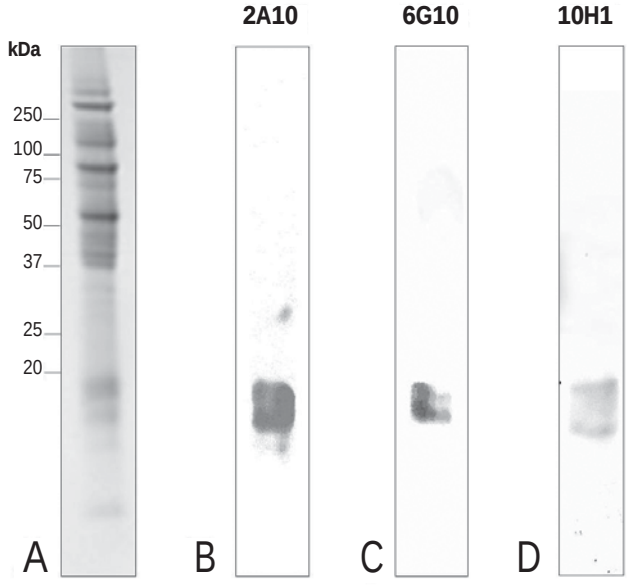

Figure 2. Sodium dodecyl sulfate polycrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis of Pla l 1 allergen in crude extracts of plantain pollen. A: Coomassie blue staining of total protein. B and C: immunoblot as above, probed with the anti-Pla l 1 antibodies (2A10 and 6G10, respectively). D: immunoblots as above probed with the monoclonal antibody 10H1 to Ole e 1 allergen.

min in PBS, and incubated in detection buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$, 1mM levamisole) for 5 min. The color reaction was left to develop overnight using nitro blue tetrazolium choloride / 5-bromo-4-chloro-3-indolyl phosphate, toluidine salt (NBT/BCIP) as substrate. Observations were made and photographs taken in a Zeiss Axioplan microscope. Negative control reactions were set by omitting the primary antibody.

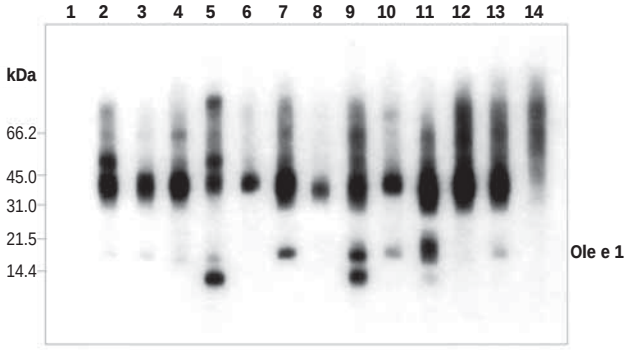

Figure 3. Immunodetection of *O europaea* pollen extracts by using sera of patients sensitized to *P lanceolata* pollen. Lane 1, negative control using serum from a nonallergic individual.

For transmission electron microscopy studies, non-specific binding sites of sections were blocked in 5% w/v BSA and 0.1% v/v Tween 20 in PBS for 15 min. Grids containing sections of English plantain anthers at the mature stage were then incubated with the 2A10 anti-Pla l 1 or the anti-Ole e 1 10H1 antibody diluted 1/50 in blocking solution for 12 h. Alternatively, sections of dehiscent olive pollen were incubated with 2A10 anti-Pla 1 antibody (1/50 in blocking solution) for 12 h. In both cases, after rinsing in PBS 3 × 20 min, grids were treated for 1 h with a goat antimouse IgG antibody coupled to 15 nm gold particles (British BioCell International, Cardiff, UK) diluted 1/50 in PBS. Finally, sections were washed with PBS and ultrapure water, and stained with uranyl acetate. Observations were made in a JEOL JEM-1011 transmission electron microscope operating at 80 kV. Negative sections were treated as described above, although blocking reagent was used instead of the primary antibody solution.

Results

Immunoblot Assays Using Monoclonal Antibodies

Crude protein extracts from olive pollen were separated by SDS-PAGE and probed with the 2A10 and 6G10 monoclonal antibodies to Pla l 1. Both antibodies cross-reacted with up to 3 protein bands in a range of 18-22 kDa (Figure 1, Lanes 3 and 4). The sizes of these bands agreed with those of the different variants of glycosylation of the Ole e 1 allergen and were clearly distinguishable after either Coomassie blue staining (Figure 1, Lane 1) or immunodetection with the 10H1 anti-Ole e 1 antibody (Figure 1, Lane 2).

When crude extracts of *P lanceolata* pollen were separated by SDS-PAGE and probed with the anti-Ole e 1 antibody 10H1 in immunoblot experiments, a faint cross-reaction was observed (Figure 2D). The size of the cross-reacting band agreed with the observed size of the Pla l 1 allergen, either in Coomassie-stained gels (Figure 2A) or after probing equivalent immunoblots with the 2A10 or 6G10 antibodies (Figure 2B and 2C, respectively).

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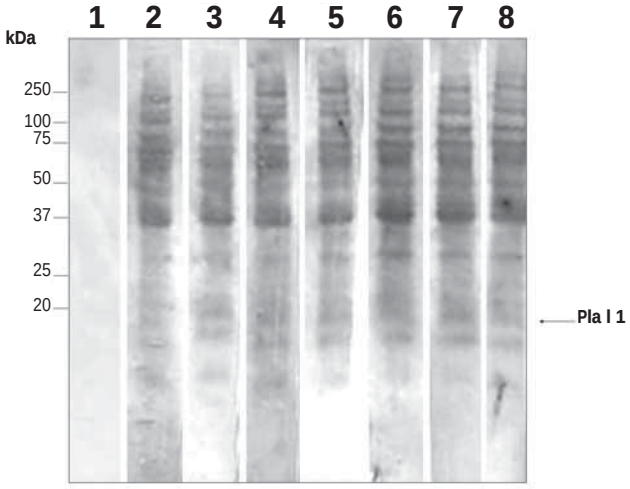

Figure 4. Immunodetection of *P lanceolata* pollen extracts by using sera from patients sensitized to *O europaea* pollen. Lane 1, negative control using serum from a nonallergic individual.

Immunoblot Assays Using Patients' Sera

Figure 3 illustrates the reactivity of the sera from individual plantain-allergic patients to an extract from olive pollen. The immunoblot showed that only a limited number of sera recognized bands of a size similar to Ole e 1 (18-20 kDa). However, most patients' sera recognized an antigen of approximately 40-45 kDa. The reverse experiment showed that all the individual sera from olive-pollen allergic patients tested did recognize a faint band close to 20 kDa in plantain pollen extracts after immunodetection (Figure 4). Higher molecular weight bands were also recognized by most sera, particularly a conspicuous band of approximately 40 kDa. In both cases, negative controls performed using the serum of a nonallergic individual showed no reactive bands.

Light Microscopy and Transmission Electron Microscopy Immunolocalization of Pla l 1 in the Pollen Grain of P lanceolata

Mature anthers of *P lanceolata* observed using light microscopy showed numerous pollen grains which displayed 3 nuclei and abundant starch granules as the main features (Figure 5A, B). Immunodetection by light microscopy of Pla l 1 in *P lanceolata* anther sections produced specific labeling in the pollen grains, whereas other anther tissues such as the epidermis were devoid of signal (Fig. 5C, D). No major differences were observed in this localization when the 2A10 and 6G10 monoclonal antibodies were used. Slight nonspecific labeling occurred in the pollen nuclei. Negative control sections showed no signal (Figure 5E).

At the ultrastructural level, mature *P lanceolata* pollen showed characteristic features (Figure 6A, B). The mature pollen grain is tricellular, showing a vegetative cell and 2 sperm cells. A major feature of this pollen is the presence of conspicuous starch granules filling the cytoplasm of the vegetative cell. The pollen wall displays several aperture

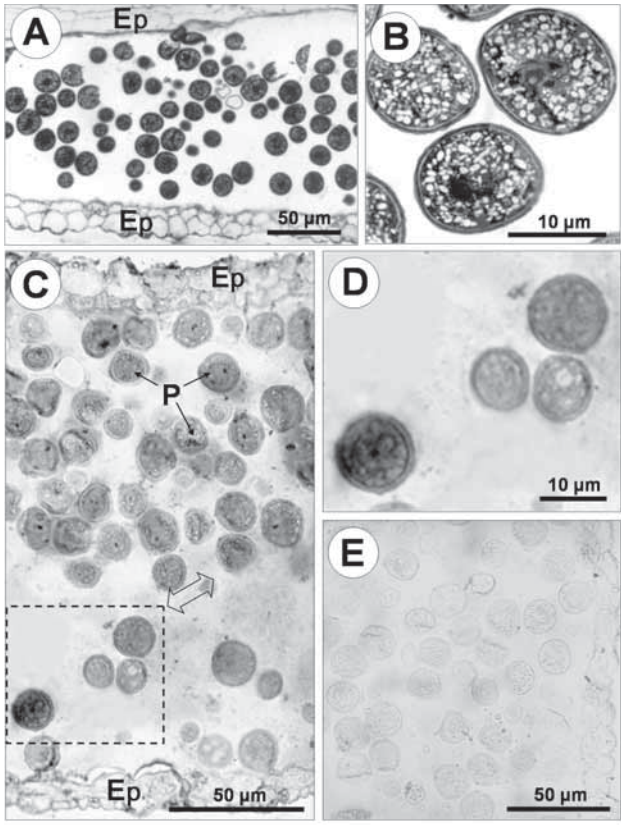

Figure 5. A-B: Light microscopy overview of a mature anther (A) and pollen grains (B) of *P lanceolata.* C-E: immunolocalization of the major allergen Pla l 1 on English plantain anther sections by using the monoclonal antibody 2A10. D: detail of C at higher magnification. E: control section treated by omitting the primary antibody. Ep indicates epidermis.

regions. After immunolocalization with the 2A10 antibody (Figure 6A), gold particles were located mainly in the cytoplasm of the vegetative cell, sometimes in association with endoplasmic reticulum cisternae, and occasionally surrounding the starch granules. Starch granules themselves were devoid of gold particles. Less significant labeling also appeared in the outer layer of the pollen wall (ie, the exine), whereas a nonspecific signal was also detected in the nucleus of the vegetative cell. Negative control sections were practically devoid of gold particles (Fig. 6B).

Transmission Electron Microscopy Cross-Immunolocalization of Pla l 1 and Ole e 1 in the Pollen Grain of O europaea and P lanceolata

Ultrastructural characteristics of *O europaea* pollen were to a large extent different from those described for *P lanceolata.* The main differences were its bicellular nature (not shown) and the presence of lipid bodies as the main storage compound in the former, as opposed to the conspicuous starch granules present in the latter (Figures 7A, B).

When the 2A10 monoclonal antibody was used to probe olive pollen sections (Figure 7A), gold particles were located

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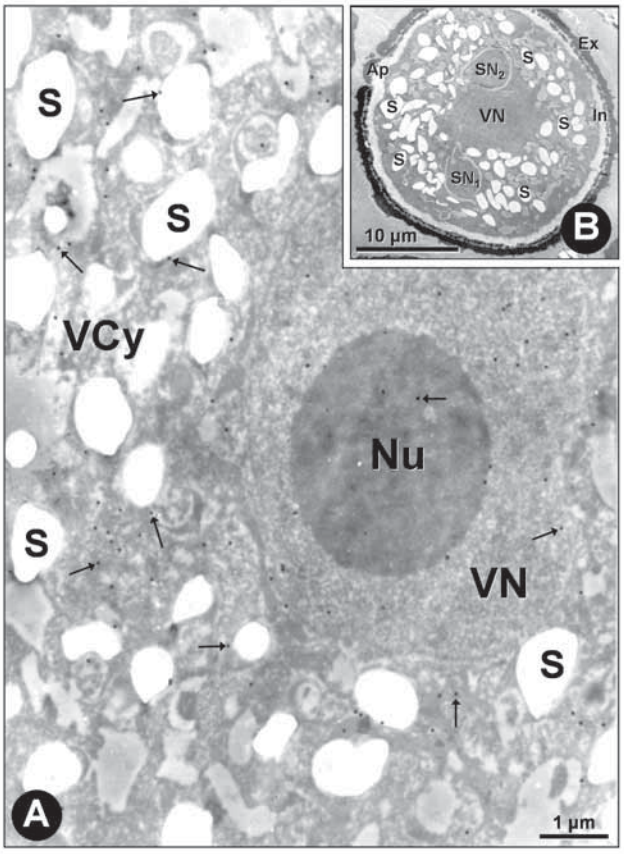

Figure 6 A: Transmission electron microscopy immunolocalization of the major allergen Pla l 1on plantain anther sections by using the monoclonal antibody 2A10, and B: Transmission electron microscopy overview of *P lanceolata* mature pollen. The vegetative cell and 2 sperm nuclei are clearly distinguishable. Numerous starch granules are visible scattered in the cytoplasm of the vegetative cell. Gold particles (arrows) are mainly located in the cytoplasm of the vegetative cell. Control was by omitting the primary antibody (data not shown). Ap: aperture, Ex: exine, SN_1: sperm nucleus 1, SN_2: sperm nucleus 2, In: intine, Nu: nucleolus, S: starch, VCy: vegetative cell cytoplasm, VN: vegetative nucleus.

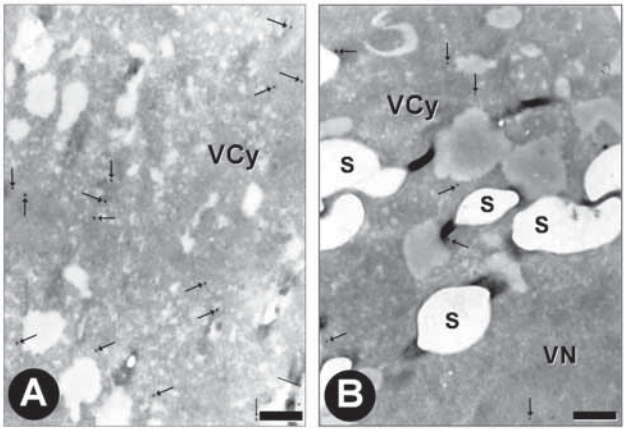

Figure 7. A: cross-reactivity of the antibody 2A10 in olive mature pollen sections (cv. Picual). B: cross-reactivity of the antibody 10H1 in mature plantain pollen sections. S: starch, VCy: vegetative cell cytoplasm, VN: vegetative nucleus. Bars= 4µm.

in the cytoplasm of the vegetative cell and in both the exine and the material adhering to this outer layer of the pollen wall. The intine, the vegetative nucleus, and the whole generative cell were devoid of gold. Alternatively, when we used the monoclonal antibody 10H1 to localize cross-reacting proteins in sections of *P lanceolata* pollen, labeling was significantly lower than with the 2A10 antibody. Gold particles were detected in both the cytoplasm and the nucleus of the vegetative cell, and in the cytoplasm of the generative cell (Figure 7B), whereas no labeling was found in the starch granules. Controls did not show significant gold labeling in any case (data not shown).

Discussion

To date, approximately 100 proteins from different sources share common sequences with Ole e 1. These proteins have been indexed as members of the so-called pollen proteins of the Ole e 1 family (Accession number: PF01190) in the Pfam protein families database [23]. These plant pollen proteins, whose biological function is not yet known, are structurally related. They are most probably secreted and consist of about 145 residues. Six cysteines are conserved in the sequence of these proteins and these seem to be involved in disulphide bonds. It has been suggested that the molecular functions of allergens related to protein turnover or stability may directly or indirectly contribute to allergenicity [24]. Although members of this group show sequence similarity with soybean trypsin inhibitor-related proteins, no conclusive evidence of this putative relationship has been established to date. Within this family, Ole e 1 has shown a relatively high level of cross-reactivity with similar allergenic proteins present in the pollen of other species from the *Oleaceae* family including ash, privet, and lilac [2, 25, 26]. Structural similarity is also present in other non-*Oleaceae* allergens such as Lol p 11 from ryegrass (*Lolium perenne*) [27], Che a 1 precursor from *Chenopodium album* [28], and Phl p 11 from common timothy (*Phleum pratense*) [29]. With the exception of the Pla l 1 allergen from *P lanceolata* shown in this paper, the extension of IgE cross-reactivity between Ole e 1 and the corresponding homologues in these plants is not clear.

In the present study, the antigenic relationship between Pla l 1 and Ole e 1 was investigated by means of different immunochemical techniques. The results confirm the presence of common epitopes in both allergens, which are recognized to a greater or lesser extent by these antibodies.

The presence of isoforms and variants of a given protein is a common feature within other members of the "pollen proteins of the Ole e 1 family" such as Phl p 11. As for Pla l, nucleotide sequence polymorphism and the presence of 3 isoforms have been reported [18]. Further characterization of the cross-reactivity of each isoform could help to define the epitopes involved in the interaction.

IgE cross-reactivity between both allergens was also confirmed using patients' sera. The analysis of these crossed allergograms showed that, in most cases, neither Ole e 1 nor

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Pla l 1 is the only, or even major cause of the cross-reaction of patients' sera, unlike the protein components of higher molecular weight in both extracts. The conspicuous cross-reactive bands of approximately 40 kDa present in both the olive and plantain pollen extracts, which are recognized by most patients' sera, may well correspond to a 1,3-β-glucanase. The Ole e 9 allergen from olive pollen (46 kDa) is a 1,3-β-glucanase and a highly prevalent allergen [30]. Most of the 1,3-β-glucanases characterized to date belong to a family of proteins with a molecular mass of 33-36 kDa. However, a very limited number of larger 1,3-β-glucanases (42-46 kDa) with an extension at the C-terminal end has been reported. The ubiquitous presence of 1,3-β-glucanases in latex, vegetable foods, and, more recently, in pollens suggests that they could be a novel type of panallergen able to induce cross-reactivity [30,31]. However, their contribution to cross-reactivity between olive and plantain pollens should be specifically addressed in future studies.

In a previous work, 2 out of 5 anti-Ole e 1 monoclonal antibodies gave a positive cross-reactive response with Pla l 1, although this was significantly lower than with Ole e 1, suggesting the existence of related epitopes in both proteins [18]. Nevertheless, when Ole e 1 protein was probed in an enzyme-linked immunosorbent assay (ELISA), the 2A10 and 6G10 antibodies were not able to bind to this allergen. Ole e 1 was able to inhibit by up to 25% the binding of IgE to Pla l 1 after inhibition by ELISA using a pool of sera from plantain-allergic patients. However, the Ole e 1 concentration needed to reach this inhibition percentage was about 1000-fold higher than that of Pla l 1 [18].

Surprisingly, we found that 2A10 and 6G10 were able to recognize the different variants of Ole e 1, suggesting that the cross-antigenicity between Ole e 1 and Pla l 1 could be more significant than previously stated. Several factors could explain these relative divergences. Firstly, ELISA was performed using the antibodies at concentrations optimized to detect Pla l. Therefore, the negative result may be due to the lower affinity of the antibodies for Ole e 1 and conformational changes upon adsorption of the antigen to the ELISA plates, which reduced the accessibility of the epitopes. Secondly, our experiments were conducted under denaturing conditions, whereas ELISA was carried out using native proteins. Denaturing might lead to changes in both the antigenic and allergenic properties of allergens. Although most of the antigenic epitopes of Ole e 1 have been shown to have a conformational nature [32], our results suggest that 2A10 and 6G10 antibodies recognize continuous epitopes of Ole e 1.

As for their subcellular localization, both proteins localize equivalently, taking into account the relevant differences emerging from the bicellular and tricellular nature of olive and plantain pollens, respectively. Detailed studies have shown that the synthesis and storage of Ole e 1 takes place in the endoplasmic reticulum of the vegetative cell [33]. In addition, the allergen has been found in the outer layer of the pollen wall (exine) and the tapetum, which has also been considered a possible origin of the allergenic proteins present in the pollen wall [34], and Ole e 1 is released into the culture medium throughout germination [35]. These results support the idea of rapid Ole e 1 diffusion in isotonic media, an important feature which is generally assumed to be necessary for a protein to be considered a major allergen. The present work represents the first report on Pla l 1 localization in pollen. The Pla l 1 allergen was located in the cytoplasm of the vegetative cell, which is also frequently associated with the endoplasmic reticulum. The presence of only limited labeling in the exine might be explained by the fact that pollen samples were processed using conventional fixation techniques. Only the use of cryotechniques can guarantee good preservation of the antigenicity of samples and prevent washing out of the soluble protein by diffusion to the aqueous fixative [33]. The cross-localization experiments reported in this paper, which match the localization of the noncrossed antibodies, are additional evidence of the presence of common epitopes in both proteins. The localization reported for Pla l 1 agrees with the hydrophilic nature of this protein [16]. The structural similarities reported for Ole e 1 and Pla l 1 and their similar localization might account for a shared biological function in the pollen grain, which should be further explored. Both allergens share common epitopes, which can be cross-recognized by different antibodies and patients' sera, although this antigenic similarity seems to have little clinical relevance.

Acknowledgments

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