

OLIVE TREE GENETIC BACKGROUND IS A MAJOR CAUSE OF PROFILIN (OLE E 2 ALLERGEN) POLYMORPHISM AND FUNCTIONAL AND ALLERGENIC VARIABILITY

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INTRODUCTION

Profilin was first identified in plants as birch allergen (Valenta et al. 1991). Plants have several genes encoding highly divergent profilin isoforms (Kovar et al. 2000), differentially expressed, and with biochemical and functional diverse functions (Huang et al. 1996). Structurally, overall look of plant profilins is similar to these from yeast and vertebrate, although the identity of primary amino acid sequence is only about 30% (Thorn et al. 1997), which implicate profilins in key conserved functions throughout different kingdoms. The in vitro biochemical data suggested that different profilin isoform functions distinctly (Kovar et al. 2000), which supports an isovariant dynamics model, where particular isoforms have differential functions/activities. Supporting this idea, it has been proposed that plant profilin family multi-functionality might be inferred by natural variation through profilin isovariants generated among germplasm, as a result of polymorphism. The high variability might result in both differential profilin properties and differences in the regulation of the interaction with natural partners, suggesting that isovariant dynamics may expand the responses of actin cytoskeleton or buffer it to against stress (Jimenez-Lopez et al. 2012).

Profilin is a major regulator of actin dynamics and is crucial for cellular growth, morphogenesis and cytokinesis. In addition to binding to actin, profilins bind to other partners like stretch of poly-proline (PLP) and proline-rich proteins, and phospholipids. The proline-binding ability could be a major function, being different among profilin isoforms, affecting actin-based structures (Kovar et al. 2001).

The binding of profilin to phospholipids links its potential role to vesicle trafficking (Janssen and Schleicher 2001), in addition to acting at critical points of signaling pathways initiated by events in the plasma membrane and transmitted by transduction cascades to promote cytoskeletal rearrangements (Baluska et al. 2002).

Several locations have been attributed to profilin. They have been localized in different plant cells and tissues, including the microspores, pollen grains and pollen tubes (Kandasamy 2002). Plant profilin was reported to be localized in the cytoplasm of pollen tube uniformly (Vidali & Hepler 1997), however, no clear picture has yet been established about the precise location of profilin in the pollen tubes.

In the present study, we have used two experimental approaches, immune-reactivity in blotting experiments, and immunogold experiments for profilin cellular localization at TEM, with the aim of analyzing the differential immune-reactivity of profilins as result of their high sequence polymorphism, which also drives profilins the subcellular locations.

MATERIAL AND METHODS

Plant material

Olive (*Olea europaea* L.) pollen was collected during May and June from olive trees of Picual cultivar, grown in the olive germplasm collection of the Estación Experimental del Zaidín, CSIC, Granada, Spain.

In vitro olive pollen germination

Mature pollen was in vitro germinated as described by Morales et al. (2008).

Proteins extraction

Mature pollen samples were re-suspended in an extraction buffer (PBS), pH 7.4 to a proportion of 5 mL solution per 0.5 gram of fresh tissue and then incubated at 4°C for 4 h with vigorous shaking. After centrifugation at $13000 \times g$ for 30 min at 4°C, the supernatants were removed, dispensed into aliquots and stored at -20°C. The protein concentration was measured following the Bradford (1976) method. In total, 30 µg of protein per lane was loaded into a 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel (PAGE), as described by Laemmli (1970).

Protein transference and immunoblotting

After completion of proteins separation by SDS-PAGE, they were transferred onto polyvinylidenedifluoride (PVDF) membranes at 65 V for 1h as described by Towbin et al (1979). For immunodetection, blots were incubated for 4 h at 25°C with a blocking solution containing 0.1% Tween 20 and 10% dried skimmed milk in Tris-buffered saline (TBS). The membranes were then probed with:

1. Whole olive and maize profilins polyclonal antibodies anti-ZmPRA (dilution 1:500) and anti-Ole e 2 (dilution 1:20000), respectively).
2. Polyclonal antibodies specific to particular isoforms of maize profilin like ZmPRO5, ZmPRO4 and ZmPRO3, in a dilution 1:500.

A peroxidase-conjugated anti-rabbit IgG (Promega Co) diluted 1:10000 served as the secondary antibody, and the detection reaction was developed using NBT-BCIP colorimetric system.

Transmission Electron Microscopy

The germinated pollen grains were treated as described in Morales et al. (2008) for immune-electron microscopy analysis, by using the above specific antibodies.

RESULTS

Profilin olive cultivars differential reactivity characterization

The analysis of reactivity among cultivars against individual antiserum showed significant differences between Leccino and Picual, Lechin de Sevilla and Picudo, Lucio and Frantoio, Blanqueta and Farga, as well as between Picudo and Cornicabra corresponding to the immunoblots individually probed with anti-PRA, anti-ZmPRO3, anti-ZmPRO5, anti-ZmPRO4 y anti-Ole e 2, respectively (Figure 1A-E). Moreover, in the other way around, it is possible to observe clear differences between antisera for a defined cultivars, such as Hojiblanca, Arbequina, Cornicabra, Bella de España and Empeltre for the immunoblotting corresponding to anti-ZmPRO3; Lechin de Sevilla, Verdial de Huevar, Loaime, Lucio and Leccino for anti-ZmPRO4; and Sourani and Villalonga for anti-Ole e 2 (Figure 1A-E).

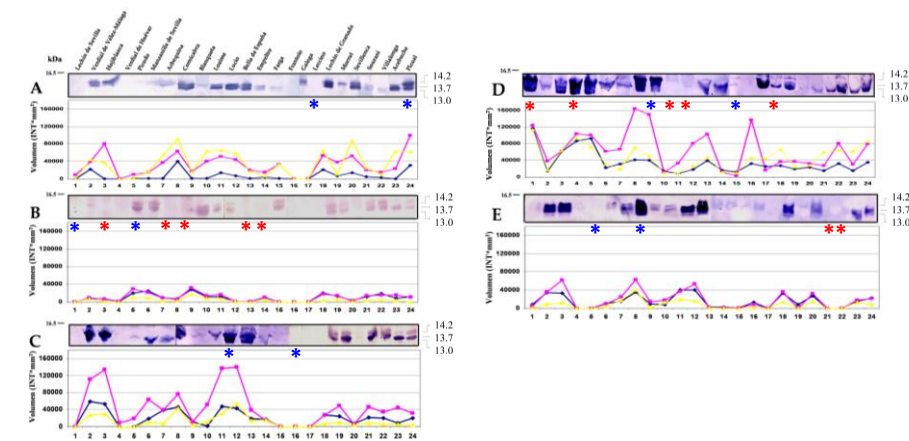


Figure 1. Immuno-reactivity analysis of profilin from 24 olive pollen cultivars. Reactivity of protein crude extracts from 24 olive cultivars were assayed against different maize profilin antisera, a) anti-PRA, b) anti-ZmPRO3, c) anti-ZmPRO5, d) anti-ZmPRO4, e) as well as against olive profilin antiserum anti-Ole e 2. It was appreciable up to 3 reactive bands about 13, 13.7 and 14.2 kDa, corresponding to different isoforms of profilins. The intensity of the reactive bands was quantified by a densitometric analysis: yellow plot corresponding to 13kDa bands, pink (13.7 kDa) and blue (14.2 kDa). Red asterisks highlighted the differential reactivity (very high or very low) of defined cultivars to different antisera, whereas blue asterisks highlighted the differential reactivity among cultivar to defined antisera.

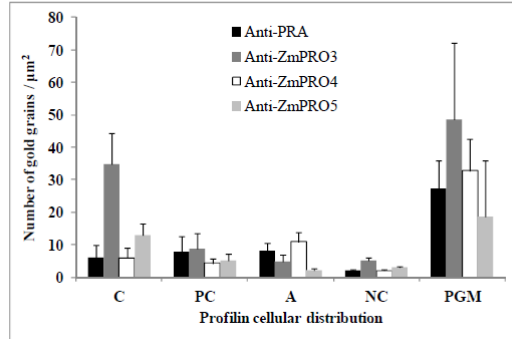
Profilin cellular localization

All immuno-localizations showed that profilins were preferentially located in the cytoplasm, in addition to both nuclei of vegetative/generative cell. Moreover, abundant gold grains were located in the pollen apertures, along the pollen wall, pollen tube and the pollen tip, as well as in the material associated with the pollen grain exine (pollen coat). No significant number of labeling were found in the negative controls, for which were omitted the primary antiserum. The overall number of gold grains in the sections was variable, and depending on the antiserum used.

In order to determine differences in the gold grain distribution for the different antisera used as markers for profilin isoforms, we proceeded to count the gold particles present on each of the above mentioned compartments. The results of this quantification are showed in the table 5. The most abundant immune-labeling was observed in sections incubated with individual antiserum following the next order: anti-ZmPRO3 > ZmPRO4 > ZmPRO5. Antisera anti-ZmPRO5 and ZmPRO3 showed a preferential cytoplasmic immunolocalization, whereas anti-PRA and anti-ZmPRO4 showed a preferential localization in the apertural regions of the pollen grain.

Table 1. Gold grains count for each antiserum used in this study. Measures corresponded to gold grains/μm². Profilin counting values corresponding to the different subcellular localization were plotted. C= Cytoplasm; PC= Pollen Coat; A= Aperture; NC= Negative Control; PGM= Pollen Germination Media.

Antiserum	Profilin distribution				
	Cytoplasm	Pollen Coat	Aperture	Negative Control	Pollen Germination Media
Anti-PRA	6.06±3.94	7.83±4.67	8.22±2.22	2.00±0.42	27.13±8.89
Anti-ZmPRO3	34.70±9.66	8.83±4.54	4.86±2.04	5.00±0.87	48.56±23.76
Anti-ZmPRO4	5.91±3.02	4.34±1.32	10.83±3.06	2.00±0.38	32.75±9.63
Anti-ZmPRO5	13.00±3.35	5.14±1.95	2.20±0.45	3.00±0.44	18.70±17.36



DISCUSSION

Profilins as pan-allergens are present in a wide variety of plant sources, and responsible for numerous cross-reactions. On the other hand, profilins are also able to elicit allergic responses highly specific by recognition of specific epitopes (immune-dominant regions of recognition and interaction with B cells and T of the human immune system). However, it has been shown that the cross-reactivity also attends through humoral responses mediated by T cells, i.e. reactivity to allergens of plant foods (Mal d 1, Api g 1 and Dau c 1) with the pollen allergen Bet v 1. The "double allergenic activity" of profilin can be explained by the combination of a high structural conservation, together with the presence of a high sequence polymorphism.

The experimental results clearly demonstrate that different forms of profilins have described differential immunological characteristics as they respond differently to the antibodies used. This suggests that the recognition of profilins by the human immune system would also very likely to be differential. Several reasons can justify the broad cross-reactivity of the different profilins and the polymorphism seems to be one of the most plausible. Polymorphism is a common feature in many allergenic proteins. In olive, it has been shown that polymorphism of the allergen Ole e 1 is clearly linked to genetic background (cultivar) (Hamman-Khalifa et al. 2008), similarly to what happens to Ole e 2, where there are differential molecular characteristics due to polymorphism, which would be sufficient to explain the differences in reactivity allergenic / immunogenic among profilins from different species, different olive varieties, and even among the same isoforms of profilins (Jimenez-Lopez et al. 2012).

The experimental data suggest that the profilin family of proteins likely contains numerous functionally-distinctive isoforms, also reflected in differential cellular localizations as a result of a differential expression of some forms of profilins in vitro germination of pollen grain, and the preferential localization of some forms of profilins in different cellular compartments. These data also revealed that the differential immune-reactivity of profilins is likely the result of the presence of both common and specific epitopes features, which would be generated by the described sequence polymorphism, and might explain differential sensitizations of allergenic patients to olive pollen cultivars as well as cross-reactions between pollens from different species, as well as pollen and food allergens.

In the present work, it has been identified up to three immune-reactive bands to antibodies in the different extracts analyzed, after separation of the polypeptides by electrophoresis. The number of bands identified in other studies (Alché et al. 2007) also ranges from 3 to 5, depending on the separation methods employed, and the observed molecular weight ranges are very similar. The observed differences in the reactivity of the extracts of different varieties of olive, seems to support the varietal character as discriminatory parameter in pollen allergens, as clearly was demonstrated for other allergens such as Ole e 1 (Hamman-Khalifa et al. 2008), and Ole e 2 (Jimenez-Lopez 2008; Jimenez-Lopez et al. 2012) in the case of olive.

Cross-reactivity between profilins has broad implications in the phenomena of allergy, being responsible for many cases of double sensitization to pollens and various foods. Furthermore, the high cross-reactivity might justify the current use of a single profilin (recombinant profilin of birch pollen, Bet v 2) for the diagnosis of allergy. The existence of high polymorphism and differential reactivity to different profilin isoforms may have a number of consequences for the diagnosis and allergy therapy. Given the differential reactivity of patients to different forms of profilins, it is extremely important that the extracts used in clinical trials should take in consideration the existence of polymorphism in these molecules. As reviewed by Alché et al. (2007), the content of allergens in the protein extracts should be as similar as possible to the panel of proteins to which the atopic patient is usually exposed and reactive. Therefore, in the case of patients with allergy to profilins, it should be carefully analyzed their reactivity to the different isoforms, in order to adjust or "personalize" the treatment. In addition, a great advantage of this customization is the increased safety of immunotherapy treatments, avoiding undesirable sensitization induced "de novo" by immunotherapy, which have been documented by several authors.

New concepts in diagnosis and therapy often include the use of recombinant allergen molecules. Recombinant allergens undoubtedly provide tremendous advantages over the use of specific conventional allergen immunotherapy, based on the use of extracts from natural sources. However, a reduction in the number of allergen proteins in the extracts for immunotherapy (as is happening through the exclusive use of a single recombinant profilin form) may lead to the emergence of substantial differences between vaccines and the actual exposure of patients to their environments, unless a careful selection of the panel of recombinant allergens for immunotherapy is made. This strategy can be incorporated into virtually all new vaccines currently under development to improve the diagnosis and therapy, and to include the hybrid or modified molecules, allergen fragments, multimers, or the design of hypoallergenic proteins. For instance, a detailed reactivity analysis of isoforms present in particular cultivars, combined with protein sequence analysis, could aid the design of hypoallergenic proteins, which might complement the strategies currently in use (Marazuela et al. 2007). Besides a thorough investigation of the allergenic isoforms of the germplasm species could also help identifying natural hypoallergenic profilin isoforms in some cultivars of olive.

CONCLUSION

The polymorphism in profilin isovariants is reflected in the differential immune-reactivity exhibited by different cultivars to antibodies generated against specific profilins, in addition to differential subcellular location in pollen grains and germinated pollen tubes. Both characteristics lead to strongly propose that functional differences among profilin isoforms, as well as regulatory pathways throughout profilin-ligand binding properties, could

have a direct influence in the subcellular location and actin cytoskeleton dynamics as direct consequence of polymorphism. Furthermore, this variability reflected in the epitopes generation in panallergen like Ole e 2, has extreme importance in the standardization of formulations for allergy diagnosis in clinical trials and tailored-immunotherapy development.

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