

Ca²⁺-binding allergens from olive pollen exhibit biochemical and immunological activity when expressed in stable transgenic *Arabidopsis*

Amalia Ledesma¹, Verónica Moral², Mayte Villalba¹, Julio Salinas² and Rosalía Rodríguez¹

¹ Dpto. Bioquímica y Biología Molecular I, Universidad Complutense, Madrid, Spain

² Dpto. de Biotecnología, Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria, Madrid, Spain

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Correspondence

R. Rodríguez, Departamento de Bioquímica y Biología Molecular I, Facultad de Ciencias Químicas, Universidad Complutense, 28040 Madrid, Spain
Fax: +34 913 944159
Tel. +34 913 944260
E-mail: rrg@bbm1.ucm.es.

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Employing transgenic plants as alternative systems to the conventional *Escherichia coli*, *Pichia pastoris* or baculovirus hosts to produce recombinant allergens may offer the possibility of having available edible vaccines in the near future. In this study, two EF-hand-type Ca²⁺-binding allergens from olive pollen, Ole e 3 and Ole e 8, were produced in transgenic *Arabidopsis thaliana* plants. The corresponding cDNAs, under the control of the constitutive CaMV 35S promoter, were stably incorporated into the *Arabidopsis* genome and encoded recombinant proteins, AtOle e 3 and AtOle e 8, which exhibited the molecular properties (i.e. MS analyses and CD spectra) of their olive and/or *E. coli* counterparts. Calcium-binding assays, which were carried out to assess the biochemical activity of AtOle e 3 and AtOle e 8, gave positive results. In addition, their mobilities on SDS/PAGE were according to the conformational changes derived from their Ca²⁺-binding capability. The immunological behaviour of *Arabidopsis*-expressed proteins was equivalent to that of the natural- and/or *E. coli*-derived allergens, as shown by their ability to bind allergen-specific rabbit IgG antiserum and IgE from sensitized patients. These results indicate that transgenic plants constitute a valid alternative to obtain allergens with structural and immunological integrity not only for scaling up production, but also to develop new kind of vaccines for human utilization.

Allergens are proteins or glycoproteins present in different biological sources that initiate IgE-mediated allergic reactions in hypersensitive patients [1]. IgE antibodies are able to facilitate the activation of effector cells within the immune system that leads into mediators release responsible for the allergy-related symptoms. Allergen-specific immunotherapy is the current preventive treatment method and represents a unique curative approach [2,3]. This treatment involves the presence of nonallergenic and sometimes toxic components, which may result in the undesirable

occurrence of systemic reactions in patients. Moreover, because the standardization for several allergens is a difficult task, natural extracts do not assure a reproducible allergen composition. A promising alternative to classical protocols is the use of well-defined recombinant allergens. Genetic engineering has allowed the production of a high number of allergens [4]. The main advantages of this technology are the large amounts of available protein and the possibility to modify their allergenic properties by site-directed mutagenesis. However, the availability of recombinant allergens has

Abbreviations

AtOle e 3, recombinant Ole e 3 produced in *Arabidopsis thaliana*; AtOle e 8, recombinant Ole e 8 produced in *Arabidopsis thaliana*; CaBP, Ca²⁺-binding protein; CaMV 35S, cauliflower mosaic virus 35S; nOle e 3, natural Ole e 3, isolated from olive pollen; rOle e 3, recombinant Ole e 3 produced in *Escherichia coli*; rOle e 8, recombinant Ole e 8 produced in *Escherichia coli*.

allowed the knowledge of 3D structures which helps to accurately define putative IgG and IgE epitopes.

Expression of allergen-specific DNAs is now possible in a variety of prokaryotic and eukaryotic host organisms. The bacteria *Escherichia coli* has been the system most commonly used because of it is well-characterized genetically, it has the ability to grow rapidly and it is easy and nonexpensive to handle. However, it lacks post-translational machinery which has led to the alternative employment of eukaryotic cells such as yeast, baculovirus/insect, plants or mammalian cells. In recent years, plant-based expression systems have generated great interest and expectation because of the possibility to produce edible vaccines [5,6]. The absence of microorganism-derived toxins and avoidance of the continuous injections that patients receive in classical immunotherapy protocols make this plant-based vaccine technology an attractive option. Other advantages of this expression system are the low cost of raw materials, rapid scale-up and, especially for proteins from vegetable sources, the ability to carry out post-translational modifications, including the glycosylation pathway of higher eukaryotes. Several antigens of high clinical significance such as the cholera toxin B subunit, immunoglobulins, α -interferon, VP1 protein from foot-and-mouth disease virus, or glycoprotein S from transmissible gastroenteritis virus have been expressed in transgenic plants or by means of plant viruses [5,6]. Interest in recombinant production in plant-derived systems has been extended to the field of allergies. Thus, some allergens such as Bet v 1 [7], Mal d 2 [8], Hev b 1 and Hev b 3 [9] have been transiently produced in *Nicotiana benthamiana* using plant viral vectors. Stable rice transgenic plants have been recently used to produce T-cell epitope peptides of Cry j 1 and Cry j 2 allergens from Japanese cedar fused with a storage protein from rice seeds [10], but the production of whole allergens has not been explored to date.

In Mediterranean countries and some parts of North America, olive pollen is one of the main causes of pollinosis [11,12]. This pollen contains a complex mixture of allergenic proteins, from which 10 allergens have been isolated and characterized to date (Ole e 1 to Ole e 10) [13,14]. Two of these allergens, Ole e 3 and Ole e 8, belong to the widespread family of Ca^{2+} -binding proteins (CaBPs). Both proteins possess in their sequences the structural EF-hand motif, composed of 12 conserved amino acid residues directly implicated in the binding of the protein to calcium ions. Ole e 3 [15,16] is a panallergen member of a pollen-specific family of small CaBPs called polcalcins which contain two EF-hand motifs and are responsible

for cross-reactivity among pollens [17]. Ole e 8 is a 19 kDa protein with four EF-hand motifs [18]. Putative homologous allergens to Ole e 8 have been identified in the *Oleaceae* family and juniper [19]. When expressed in *E. coli*, these olive allergens maintained their biochemical capacity to bind calcium ions [16,18].

Here, we report the expression of Ole e 3 and Ole e 8 in transgenic plants of *Arabidopsis thaliana*, and also characterization of the biochemical and immunological properties of the recombinant products and their comparison with their olive pollen and/or *E. coli*-produced counterparts. Our results show that transgenic plants constitute a suitable alternative for producing allergens with structural and immunological integrity for both clinical and scientific purposes, as well as for developing new ways of vaccination.

Results

Obtaining *Arabidopsis* transgenic plants containing Ole e 3 and Ole e 8 cDNAs

Binary pROK2-OLEE3 and pROK2-OLEE8 plasmids carrying the Ole e 3 and Ole e 8 cDNAs, respectively, were obtained by subcloning the corresponding cDNAs from previously obtained constructs [16,18] into the binary pROK2 plasmid (Fig. 1). pROK2 uses the cauliflower mosaic virus 35S (CaMV 35S) promoter for nominally constitutive transcription of the cloned genes [20]. Recombinant pROK2 plasmids allow stable integration of T DNAs into plant nuclear chromosomal DNA and a selection of transformants on kanamycin-containing medium.

Arabidopsis Col plants were transformed with pROK2 recombinant plasmids as described in

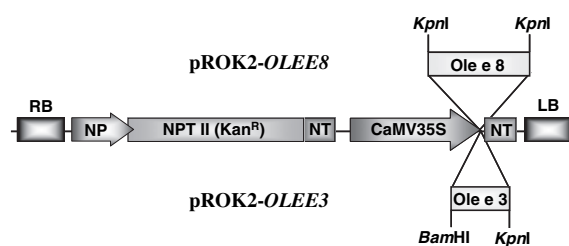


Fig. 1. Schematic structure of binary plasmids pROK2-OLEE3 and pROK2-OLEE8 used for *Agrobacterium*-mediated transformation. The cDNA sequences corresponding to Ole e 3 and Ole e 8 allergens were cloned downstream of the CaMV 35S promoter in pROK2 plasmids followed by the nopaline synthase terminator (NT). These plasmids contain the NPTII gene between the nopaline synthase promoter (NP) and the NT, as well as the left (LB) and right (RB) borders of transferred DNA that demarcate the sequences which are integrated into the plant genome.

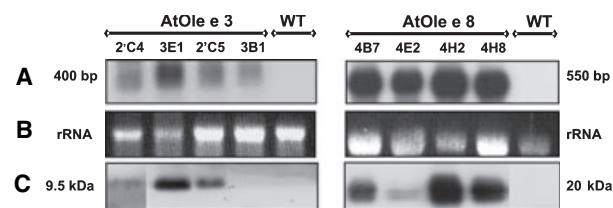


Fig. 2. Expression analysis of Ole e 3 and Ole e 8 in transgenic *Arabidopsis* plants. (A) Northern blot hybridizations of total RNA from four independent transgenic *Arabidopsis* lines and from wild-type plants (WT) with radiolabelled specific probes for each allergen. The resulting size of the bands is indicated. (B) Ethidium bromide staining of rRNA to assess the integrity of samples and loading. (C) Western blot analysis in SDS/PAGE of total protein extracts from the transgenic *Arabidopsis* lines and wild-type plants (WT). A specific-polyclonal antiserum raised against Ole e 3 or Ole e 8 was employed. The resulting size of the band is indicated.

Experimental procedures by *Agrobacterium*-mediated transformation, and more than 50 lines of transformants containing each construct were isolated and self-pollinated to obtain T₂ and T₃ generations. Ten independent T₃ lines homozygous for a single copy of each transgene were selected for further analysis. In all cases, transgenic plants were phenotypically similar to wild-type untransformed *Arabidopsis*.

Analysis of allergen expression in *Arabidopsis* transgenic plants

Analyses of RNA expression were performed by northern blot hybridizations using total RNA from independent transgenic lines and a specific DNA probe for each allergen. A band around the predicted size was visualized in the transgenic lines (400 and 550 bp in Ole e 3 and Ole e 8 transformants, respectively), whereas no signal was observed in the wild-type controls (Fig. 2A). Figure 2B shows rRNA staining with ethidium bromide as an indication of the RNA loading in each slot.

To confirm the expression of AtOle e 3 and AtOle e 8 proteins in the transgenic lines, 50 µg of protein extract from each line was analysed by western blot immunostaining with rabbit antiserum raised against nOle e 3 or rOle e 8, respectively (Fig. 2C). The protein band detected in transgenic lines exhibited the molecular mass expected for these allergens (i.e. ~10 kDa for Ole e 3 and 20 kDa for Ole e 8). No bands were detected in the protein extract from the wild-type controls. Lines that showed the highest level of expression (3E1 and 4H2) were chosen to produce each allergen.

Quantification of AtOle e 3 and AtOle e 8 in leaves from 3E1 and 4H2 transgenic lines by means of

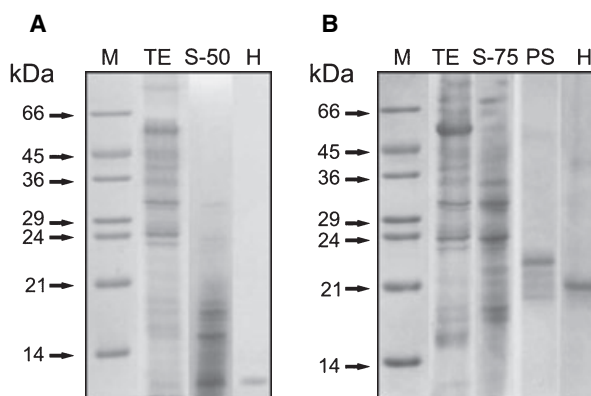


Fig. 3. SDS/PAGE analysis of the recombinant allergens. SDS/PAGE and Coomassie Brilliant Blue staining of the fraction of the eluate that contains AtOle e 3 (A) or AtOle e 8 (B) resulting after each purification step. M, molecular mass markers; TE, total protein extract; S-50, Sephadex G-50; S-75, Sephadex G-75; H, RP-HPLC; PS, Phenyl-Sepharose.

ELISA inhibition using specific polyclonal antibodies rendered percentages around 0.3% for AtOle e 3 and 0.025% for AtOle e 8 of the total soluble protein.

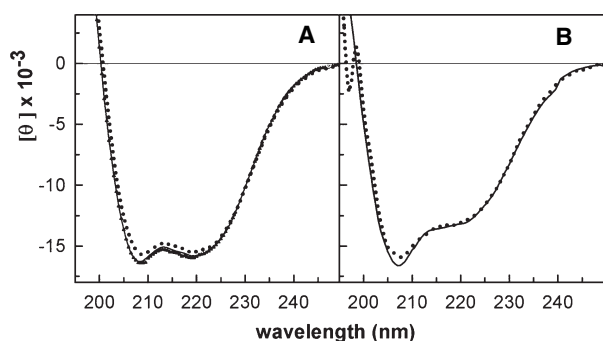
Isolation and molecular characterization of recombinant allergens

AtOle e 3 was purified using two chromatographic steps consisting of a gel-filtration chromatography on Sephadex G-50 followed by a RP-HPLC. The presence of AtOle e 3 was detected by staining with a polyclonal nOle e 3-specific antibody. SDS/PAGE and Coomassie Brilliant Blue staining of samples (0.5–50 µg of total protein, 0.5 µg in lane H) obtained from these isolation steps are shown in Fig. 3A. A single band with an apparent molecular mass of 10 kDa was visualized for the isolated protein. MS analysis of the recombinant protein gave a single peak at 9258 Da (data not shown) that agrees with the theoretical molecular mass of the allergen without the N-terminal methionine (9239 Da). The absence of this residue was confirmed by mass spectrometry after in-gel digestion of the protein with trypsin. The resulting peptides were analysed by MALDI-TOF. The molecular mass of one of these peptides fits well with the N-terminal sequence of Ole e 3 in which the methionine has been processed (Table 1). Furthermore, MS/MS analysis of this peptide confirmed the absence of this residue.

Purification of AtOle e 8 was carried out by three chromatographic steps. First, the sample was applied on Sephadex G-75. Fractions containing AtOle e 8 were loaded onto a phenyl-Sepharose column in the presence of calcium and eluted with EGTA. Finally

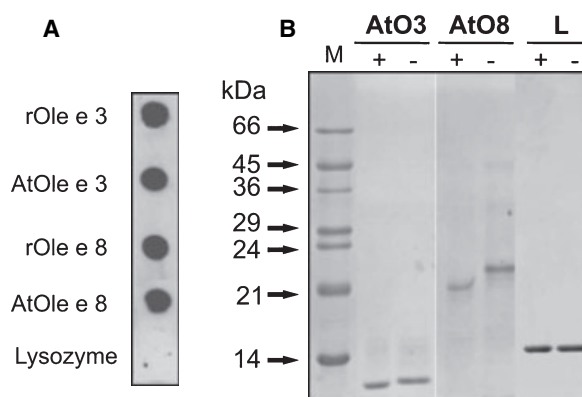
Table 1. Tryptic digestion and peptide molecular mass data. ND, not determined.

Protein	Peptide molecular mass analysis (Da)		Corresponding sequence and position	MS/MS
	Experimental	Theoretical		
AtOle e 3	1394.6	1394.6	¹ ADDPQVEVAEHER ¹² ADDPQVEVAEHER	
AtOle e 3	1782.9	1782.9	¹ ADDPQVEVAEHERIFK ¹⁵	ND
AtOle e 3	1328.7	1328.7	³⁸ TLGSVTPEEIQR ⁴⁹	ND
AtOle e 8	1692.7	1692.7	⁸³ AETDPYPSSGGENELK ⁹⁸	ND
AtOle e 8	1779.7	1779.7	¹³⁹ SVDSGDGDGYVSFEFFK ¹⁵⁴ SVDSGDGDGYVSFEFFK	
AtOle e 8	1907.8	1907.8	¹³⁹ SVDSGDGDGYVSFEFFK ¹⁵⁵	ND

**Fig. 4.** CD analysis. Far-UV CD spectra of recombinant proteins Ole e 3 (A) and Ole e 8 (B). Recombinant forms produced in *E. coli* (—), recombinant forms produced in *Arabidopsis* (●), and natural Ole e 3 obtained from the pollen (▲). Ellipticity values (θ) are expressed in units of degree cm²·dmol⁻¹.

RP-HPLC was performed. The presence of AtOle e 8 was detected by staining with a rOle e 8-specific polyclonal antibody. SDS/PAGE and Coomassie Brilliant Blue staining of the fractions resulting in each isolation step (1–50 µg of total protein, 1 µg in lane H) are shown in Fig. 3B. A single band with apparent molecular mass of 20 kDa can be visualized for the isolated protein. MS analysis of the purified protein was unsuccessful. In order to confirm the identity of isolated protein peptide-mass fingerprinting analysis was carried out. The molecular mass of the resulting tryptic peptides of Ole e 8 fit well with that expected. Thereafter, MS/MS analysis of one major peptide was performed and the resulting amino acid sequence is in accordance with that of Ole e 8 (Table 1).

Evidence for the secondary structure conformation of the isolated AtOle e 3 and AtOle e 8 proteins was obtained by comparing their CD spectra in the far UV region with those of rOle e 3 and rOle e 8 produced in *E. coli*, and natural Ole e 3 (nOle e 3) (Fig. 4). The spectra showed high ellipticity values at 208 and 220 nm, which are characteristic wavelengths of

**Fig. 5.** Ca²⁺-binding assays of AtOle e 3 and AtOle e 8. (A) Binding to ⁴⁵Ca²⁺ of recombinant *Arabidopsis*-produced allergens compared with that of recombinant *E. coli*-produced allergens. 0.5 nmol·dot⁻¹ of protein was applied. (B) Proteins were (0.5–2.5 µg) incubated in the presence (+) or absence (–) of 10 mM CaCl₂, separated by SDS/PAGE and stained with Coomassie Brilliant Blue. Lysozyme (L) was used as a negative control in both assays.

α-helical conformation. No significant differences were found between the recombinant allergens produced in *Arabidopsis* and those of the compared molecules, in terms of both the shape of the spectra and the molar ellipticity values. Therefore, it can be concluded that AtOle e 3 and AtOle e 8 are properly folded at the secondary structure level.

Calcium-binding activity of AtOle e 3 and AtOle e 8

Proteins AtOle e 3 and AtOle e 8 are able to bind radioactive Ca²⁺ to a similar extent to their recombinant *E. coli*-produced counterparts (Fig. 5A). Lysozyme was used as a negative control. Because the binding of Ca²⁺ to EF-hand proteins induces conformational rearrangements that can be detected by SDS/PAGE as a change in the mass/charge ratio, a Ca²⁺-dependent electrophoretic mobility assay was performed. As

expected, in the presence of Ca^{2+} the apparent molecular mass of AtOle e 3 and AtOle e 8 decreased by 0.3 and 2.5 kDa, respectively (Fig. 5B).

Immunological characterization of AtOle e 3 and AtOle e 8

Purified recombinant proteins were analysed by immunoblotting after separation in SDS/PAGE for their IgE-binding capacities against four olive-allergic sera with previously known reactivity for the natural or *E. coli* counterparts of these allergens [16,18]. A negative control serum was also included in the analysis. Recombinant proteins were able to bind IgE from all the olive-allergic sera but not from the negative control (Fig. 6A).

The binding capacity of the isolated proteins AtOle e 3 and AtOle e 8 to IgG from allergen-specific rabbit antiserum and to IgE from sensitized patients was also analysed using ELISA. In these experiments, *E. coli* recombinant allergens, rOle e 8 and rOle e 3, coated the wells, and AtOle e 3, AtOle e 8, rOle e 3, rOle e 8 and nOle e 3 were used as inhibitors. The same inhibition was seen whatever the origin of the allergen (Fig. 6B–E), indicating that the allergens are equivalent at the immunological level.

Discussion

Nowadays, transgenic plant technology is becoming a real alternative for the production of foreign proteins. It offers advantages over other systems such as the capacity to carry out post-translational modifications, the ability to rapidly scale-up protein production, the absence of human pathogens, and the possibility of developing edible vaccines. Recently, genetically modified rice has been used to stably express peptides from Japanese cedar allergens fused with a seed storage protein [10]. In this study, we obtained the first transgenic plants stably expressing a complete allergen. In fact, we produced the allergenic CaBPs Ole e 3 and Ole e 8 in transgenic plants of *Arabidopsis* using an *Agrobacterium*-mediated transformation system.

Arabidopsis plants were transformed with pROK2-OLEE3 and pROK2-OLEE8 recombinant plasmids, which contain the strong constitutive CaMV 35S promoter. Northern and western blot analyses of the transgenic plants showed a successful expression of Ole e 3 and Ole e 8. In these plants, the cDNAs corresponding to the allergens are stably incorporated into the plant genome, transcribed through the nuclear apparatus of the plant, and inherited by the next generations. Transgenic plants can therefore be stored as

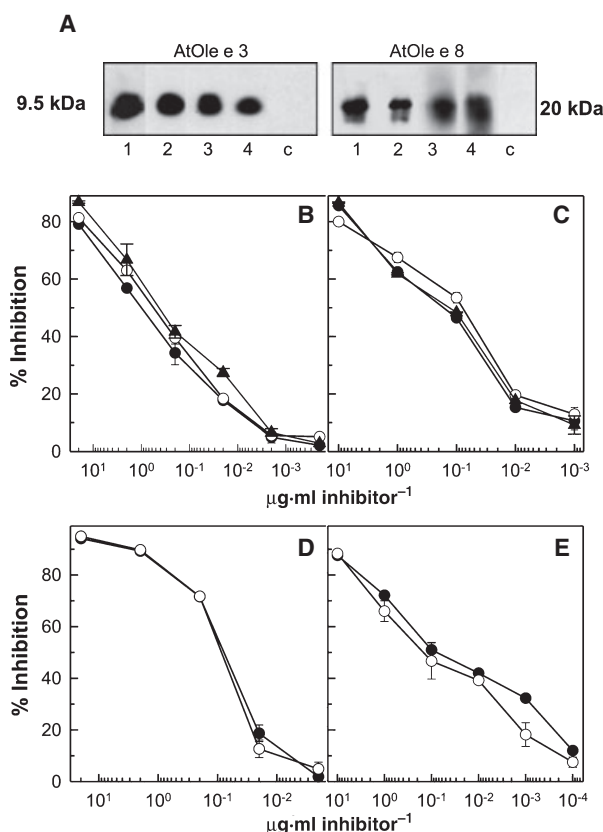


Fig. 6. Immunological characterization of AtOle e 3 and AtOle e 8. (A) Immunodetection with four (1–4) olive pollen sensitized patients' sera of purified AtOle e 3 ($1 \mu\text{g}\cdot\text{lane}^{-1}$) and purified AtOle e 8 ($1 \mu\text{g}\cdot\text{lane}^{-1}$) after SDS/PAGE and transference to membranes. c, nonallergic patients' serum control. (B–E) ELISA inhibition analysis of the binding of: (B) Ole e 3-specific polyclonal antiserum and (C) a pool of Ole e 3-sensitized patients' sera, to rOle e 3-coated wells; (D) Ole e 8-specific polyclonal antiserum and (E) a pool of Ole e 8-sensitized patients' sera, to rOle e 8-coated wells. In (B) and (C) recombinant forms of Ole e 3, produced in *E. coli* (○), in *Arabidopsis* (●) and nOle e 3 from the pollen (▲) were used as inhibitors. In (D) and (E) recombinant forms of Ole e 8, produced in *E. coli* (○) and *Arabidopsis* (●) were used as inhibitors.

seeds, which constitutes the main advantage of our system compared with the transient systems previously reported, in which allergens are expressed in plants by using plant viruses [7,8]. In addition, these plants constitute important tools to uncover the functional activities of Ole e 3 and Ole e 8, whose biological roles remain unknown.

We isolated AtOle e 3 and AtOle e 8 by means of consecutive chromatographic steps. MS analysis of the proteins, tryptic in-gel digestion followed by peptide-mass fingerprinting, as well as 'de novo' sequencing of one peptide served to identify them. Levels of foreign protein expression in transgenic plants vary greatly

depending on the polypeptide expressed and the species of host plant selected. In the case of *Arabidopsis*, levels around 0.1% of the total soluble protein have been reported previously [21,22]. We achieved expression levels of 0.3% of total protein for AtOle e 3, and lower for AtOle e 8 (0.025%). Because of the nonavailability of purified Ole e 8, we employed *E. coli*-expressed allergen, which shares immunological properties with that present in pollen extract [19], as a control to assess the molecular and immunological integrity of AtOle e 8. From the information obtained about the secondary structure of AtOle e 3 and AtOle e 8 by CD spectra, we conclude that they exhibit a high α helix content, which is characteristic of CaBPs with EF-hand motifs [23]. In fact, we show that AtOle e 3 and AtOle e 8 retain biochemical activity, because they are able to bind $^{45}\text{Ca}^{2+}$ and exhibited different electrophoretic mobility in the presence or absence of calcium ions. This capacity had been demonstrated for Ole e 3, Ole e 8 and their counterparts from *E. coli* [16,18,24]. Furthermore, AtOle e 8 displays the capability to establish interactions with a hydrophobic matrix in a calcium-dependent manner, indicating that it has a correct folding.

Immunological comparison of recombinant allergens with their natural counterparts is mandatory to establish their suitability for further clinical usage. In this way, all the sera selected for this study displayed a positive response to *Arabidopsis*-expressed forms, indicating the presence of IgE determinants in AtOle e 3 and AtOle e 8 allergens. Furthermore, inhibition analyses of the binding to IgG and IgE between both recombinant forms of each allergen (i.e. AtOle e 3 and rOle e 3, or AtOle e 8 and rOle e 8), as well as that of nOle e 3, resulted in identical inhibitory capacity, demonstrating the immunological equivalence between them. Moreover, taking into account that a dependence on the Ca^{2+} binding has been reported for the IgE responses to Ole e 3 and Ole e 8 [24], the high capability of inhibition of *Arabidopsis*-derived allergens confirms the integrity of their IgE epitopes and conformation. Considering all these results, which indicate the well-folded 3D structure and maintenance of the allergenic and antigenic epitopes for AtOle e 3 and AtOle e 8, they look like suitable molecules to be used for biochemical and clinical purposes.

An increasing amount of evidence demonstrates that plant-produced antigens can induce immunogenic responses and confer protection when delivered orally [5,6]. The potential for oral deliverance of vaccines in form of fruits, leaves or seeds highlights some important factors for patients such as the elimination of needles and a reduced medical assistance during

administration. To date, several proteins from engineered plants have been used in trials for veterinary vaccines and early phase clinical trials for human vaccination [25–27]. In the allergy field, the allergens Bet v 1 and Mal d 2 have been expressed in *Nicotiana benthamiana* via a tobacco mosaic virus vector, therefore providing a transient expression system [7,8]. In a murine model, Bet v 1 from *Nicotiana* plants generated comparable allergen-specific IgE and IgG₁ antibody responses with those obtained with rBet v 1 produced in *E. coli* [7]. *Nicotiana*-produced Mal d 2 displayed an ability to bind IgE from apple-allergic individuals, equivalent to that of the natural allergen [8]. By contrast, a genetically modified plant (*Lupinus angustifolius* L.) expressing the gene of a potential allergen (sunflower seed albumin) has been used as a vaccine that can promote a protective immune response and attenuate experimental asthma in mice [28]. Recently, a rice-based edible vaccine expressing predominant allergen-specific T-cell epitopes of Cry j I and Cry j II has been shown to induce oral tolerance in a mouse model [10]. Nevertheless, the use of transgenic plants is not the unique strategy to obtain edible vaccines. Thus, allergen Der p 5 from *Dermatophagoides pteronyssinus* has been produced in *Cucurbita pepo* L. using the zucchini yellow mosaic virus as the viral vector. Oral administration of the infected plants to mice resulted in downregulation of the synthesis of Der p 5-specific IgE, as well as of the airway inflammation [29].

Our results show that transgenic plants can be a useful as source of allergens with structural and immunological integrity, which is opening new scenarios to preventive treatments of allergy-related symptoms as well as vaccine production and delivery.

Experimental procedures

Plant material and growth conditions

Seeds of *A. thaliana* (Heynh, ecotype Columbia) were sown in pots containing a mixture of universal substrate and vermiculite (3 : 1 v/v). Pots were placed at 4 °C for 48 h in darkness to synchronize germination, and then transferred to a growth chamber set at 20 °C with a long-day photoperiod (16 h of cool-white fluorescent light, photon flux of 70 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$). Plants were irrigated with water and, once a week, mineral nutrient solution [30].

Plasmid construction and obtaining transgenic *Arabidopsis*

A 400 bp fragment corresponding to the full-length *OLEE3* was obtained from the pUC18-*OLEE3* plasmid [16] by

*Bam*HI/*Kpn*I digestion. This fragment includes the 5'- and 3'-noncoding region as well as the nucleotides that encode the protein consisting of 84 amino acid residues including the N-terminal methionine. In the case of Ole e 8, a 550 bp cDNA fragment corresponding to the coding region of a protein of 170 amino acid residues including N-terminal methionine was obtained from the pCR2.1-*OLEE8* plasmid [18] by *Kpn*I digestion. *OLEE3* and *OLEE8* cDNA fragments were subcloned in the binary plasmid pROK2 [20] under control of the CaMV 35S promoter yielding the recombinant plasmids pROK2-*OLEE3* and pROK2-*OLEE8*, respectively. Plasmids, once verified the constructs by DNA sequencing, were introduced into *Agrobacterium tumefaciens* strain C58C1 [31]. Transformation of *Arabidopsis* was performed by vacuum infiltration [32], and homozygous T₃ plants for one copy of the 35S::*OLE* transgenes were selected by segregation analysis on GM medium (MS medium supplemented with 1% sucrose) [33] containing 50 µg·mL⁻¹ kanamycin and solidified with 0.8% (w/v) agar.

Molecular biology methods

Total RNA was isolated from 4-week-old wild-type and transgenic plants according a method described previously [34]. Restriction digestions, cloning, and RNA-blot hybridizations were performed following standard protocols [35]. The Ole e 3 probe was the full-length cDNA described above. The Ole e 8 probe consisted of a 433 bp *Eco*RI fragment obtained by digestion of the full-length cDNA. RNA loading in the experiments was monitored by rRNA staining with ethidium bromide. RNA samples from each experiment were analyzed in at least two independent blots, and each experiment was repeated at least twice.

Protein extraction and purification of recombinant allergens

Leaves of transformed *A. thaliana* plants were harvested after 4 weeks, frozen at -80 °C and lyophilized. Four grams of dry and pounded material were stirred for 2 h at room temperature in 200 mL of 50 mM ammonium bicarbonate, pH 8.0 containing 1 mM phenylmethylsulfonyl fluoride. Preparations were clarified by centrifugation at 12 000 g, 4 °C for 20 min, and supernatants were filtered. Pellets of leaves were re-extracted under the same conditions for 1 h. These supernatants containing total protein extract were lyophilized and stored at -20 °C until use.

Extract containing AtOle e 3 was chromatographed on a gel-filtration Sephadex G-50 column equilibrated in 0.2 M ammonium bicarbonate. Fractions containing AtOle e 3 were lyophilized and subjected to reverse-phase HPLC in a nucleosil C₁₈ column. An acetonitrile gradient from 30 to 65% in 0.1% trifluoroacetic acid was employed for the elution of the allergen. Elution was monitored at 214 nm.

Extract containing AtOle e 8 was chromatographed on a gel-filtration Sephadex G-75 column equilibrated in 0.2 M ammonium bicarbonate. Fractions containing AtOle e 8 were lyophilized and applied onto a phenyl-Sepharose CL-4B column equilibrated in 50 mM Tris/HCl, pH 7.4, containing 0.5 mM CaCl₂. Proteins were further eluted with the same buffer containing 1 mM EGTA. A final RP-HPLC was carried out in a nucleosil C₁₈ column, with an acetonitrile gradient of 30 to 60%, in 0.1% trifluoroacetic acid. Elution was monitored at 214 nm.

Recombinant allergens rOle e 3 and rOle e 8 were produced in *E. coli* cells after 4 h of induction with isopropyl β-D-thiogalactoside and further purified as previously reported [16,18]. Natural Ole e 3 was isolated from olive tree pollen as described by Batanero *et al.* [15].

Protein concentration

Protein concentration in total extracts was determined by Lowry [36]. Purified protein concentration was determined by amino acid analysis after hydrolysis with 5.7 M HCl at 105 °C for 24 h, in sealed tubes under vacuum. Hydrolysed samples were analysed on a Beckman System 6300 amino acid analyser (Beckman Instruments, Palo Alto, CA).

Protein digestion and MS analysis

Bands of interest were manually excised from SDS/PAGE gels (see below), alkylated and digested with trypsin [37]. Bands were shrunk with 100% acetonitrile and dried. The samples were reduced with 10 mM dithiothreitol and alkylated with iodoacetamide. Finally, the samples were digested with sequencing-grade trypsin (Roche Molecular Biochemicals, Indianapolis, IN) in 25 mM ammonium bicarbonate pH 8.0. MALDI-TOF MS analyses were performed in a Voyager-DETMSTR instrument (PerSeptive Biosystems, Framingham, MA). All mass spectra were calibrated externally using a standard peptide mixture (Sigma-Aldrich, St. Louis, MO). MS/MS sequencing analyses were carried out using the MALDI-tandem-TOF MS spectrometer 4700 Proteomics Analyzer (Applied Biosystems, Foster City, CA).

CD analysis

CD spectra were obtained on a Jasco J-715 spectropolarimeter fitted with a 150 W xenon lamp [16]. Four spectra were accumulated in the far-UV region (190–250 nm) and recorded at a scanning speed of 50 nm·min⁻¹. The samples at 0.2–0.25 mg·mL⁻¹ were analysed in 0.1 cm optical-path cells in 20 mM ammonium bicarbonate, pH 8.0, at 25 °C. Mean residue mass ellipticities were calculated based on 110 and 111, respectively, to Ole e 3 and Ole e 8, as the average molecular mass/residue, obtained from the corres-

ponding amino acid composition, and expressed in terms of θ (degree $\text{cm}^2\cdot\text{dmol}^{-1}$).

Human sera and antibodies

Sera from donors with a well-documented history and symptoms of allergy to olive pollen, a positive skin test and radio-allergosorbent test class 3–6 to olive pollen extract, and with specific IgE against Ole e 3 and Ole e 8 were used. No immunotherapy had been administered to these patients. A nonallergic serum was used as a control. Written informed consent was obtained from all the individuals.

Two specific polyclonal rabbit antisera raised against nOle e 3 (isolated from pollen) [15] and rOle e 8 (produced in *E. coli*) [18] were used to follow the presence of these proteins during the purification as well as in the immunological assays.

Electrophoresis and immunoblotting

Proteins were analyzed by SDS/PAGE according to Laemmli [38] in 15% polyacrylamide gels. Between 10 and 50 μg of total protein was loaded from protein extracts or eluates; 0.5–2.5 μg was loaded when purified proteins were analysed. Proteins were either visualized by Coomassie Brilliant Blue staining or electrophoretically transferred onto nitrocellulose membranes for immunodetection, as described previously [15]. Briefly, membranes were incubated alternatively with sera from patients allergic to olive pollen (diluted 1 : 10), an Ole e 3-specific polyclonal antiserum (diluted 1 : 5000) or an Ole e 8-specific polyclonal antiserum (diluted 1 : 10 000). The binding of human IgE was detected by mouse anti-(human IgE) serum (diluted 1 : 5000; kindly donated by ALK-Abelló, Madrid, Spain) followed by horseradish peroxidase-labelled goat anti-(mouse IgG) serum (diluted 1 : 5000; Pierce Biotechnology, Rockford, IL). The binding of IgG polyclonal antiserum was detected by peroxidase-labelled goat anti-(rabbit IgG) serum (diluted 1 : 3000; Bio-Rad, Richmond, VA). The signal was developed by the ECL-western blotting reagent (Amersham Biosciences, Barcelona, Spain).

ELISA inhibition

ELISA inhibitions were performed in microtitre plates coated with 100 $\mu\text{L}\cdot\text{well}^{-1}$ of protein (1 $\mu\text{g}\cdot\text{mL}^{-1}$) as described previously [14]. Briefly, plates were alternatively incubated with a pool of sera ($n = 4$, diluted 1 : 10), a nOle e 3-specific polyclonal rabbit antiserum (diluted 1 : 30 000) or a rOle e 8-specific polyclonal rabbit antiserum (diluted 1 : 100 000), all previously incubated for 2 h at room temperature with different amounts of inhibitors. Tenfold serial dilutions from 20 $\mu\text{g}\cdot\text{mL}^{-1}$ of inhibitors for the polyclonal inhibition and from 10 $\mu\text{g}\cdot\text{mL}^{-1}$ of inhibitors for the sera

inhibition were used. The binding of human IgE and IgG polyclonal antiserum was detected as indicated above. Peroxidase reaction was developed with *o*-phenylenediamine reagent and measured as A_{492} . Each value was calculated as mean of two determinations. The percentage of inhibition was calculated according to the formula: Inhibition (%) = $(1 - (A \text{ with inhibitor} / A \text{ without inhibitor})) \times 100$.

Quantification of AtOle e 3 and AtOle e 8 production in transgenic plants was estimated by means of ELISA inhibition. Plates were coated with rOle e 3 or rOle e 8 as above described, Ole e 3- and Ole e 8-specific polyclonal antisera were incubated with known amounts of the same recombinant proteins, known amounts of total protein extracts from leaves of transgenic plants, as well as known amounts of total protein extracts from leaves of wild-type as control. Inhibition curves were represented using rOle e 3 or rOle e 8, and the amount of AtOle e 3 or AtOle e 8 contained in the total extracts was deduced from the percentage of inhibition obtained with known amounts of total protein extracts from transgenic plants.

Calcium-binding assays

Assay of Ca^{2+} binding was carried out as described previously [16]: 0.5 nmol of proteins were dotted onto a nitrocellulose membrane. After washing three times in calcium buffer (10 mM Pipes, pH 6.9, 50 mM NaCl, 0.1 mM MgCl_2), the membrane was incubated with 6 μM $^{45}\text{CaCl}_2$ (3000 mCi·mmol $^{-1}$) in calcium buffer, and washed twice with distilled water. The membrane was exposed to Agfa X-ray film for 2 h. Lysozyme was used as a negative control.

Mobility shift experiments in SDS/PAGE were performed according to Ledesma *et al.* [18] in the presence of either 10 mM EGTA, or 10 mM CaCl_2 after washing with 2 mM EGTA. Samples were stained with Coomassie Brilliant Blue.

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