An olive pollen protein with allergenic activity, Ole e 10, defines a novel family of carbohydrate-binding modules and is potentially implicated in pollen germination

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CBMs (carbohydrate-binding modules) are the most common non-catalytic modules associated with enzymes active in plant cell-wall hydrolysis. They have been frequently identified by amino acid sequence alignments, but only a few have been experimentally established to have a carbohydrate-binding activity. A small olive pollen protein, Ole e 10 (10 kDa), has been described as a major inducer of type I allergy in humans. In the present study, the ability of Ole e 10 to bind several polysaccharides has been analysed by affinity gel electrophoresis, which demonstrated that the protein bound 1,3-β-glucans preferentially. Analytical ultracentrifugation studies confirmed binding to laminarin, at a protein/ligand ratio of 1:1. The interaction of Ole e 10 with laminarin induced a conformational change in the protein, as detected by CD and fluorescence analyses, and an increase of 3.6 °C in the thermal denaturation temperature of Ole e 10 in the presence of the glycan. These results, and the absence of alignment of the sequence of Ole e 10 with that of any classified CBM, indicate that this pollen protein defines a novel family of CBMs, which we propose to name CBM43. Immunolocalization of Ole e 10 in mature and germinating pollen by transmission electron microscopy and confocal laser scanning microscopy demonstrated the co-localization of Ole e 10 and callose (1,3-β-glucan) in the growing pollen tube, suggesting a role for this protein in the metabolism of carbohydrates and in pollen tube wall re-formation during germination.

Key words: allergen, carbohydrate-binding module, 1,3-β-glucan, olive pollen, pollen germination.

INTRODUCTION

Sexual reproduction in plants is initiated when a pollen grain settles on the stigma of the style in flowers and generates the pollen tube that delivers the male gametes to fertilize the eggs [1,2]. Although the biochemical mechanisms of this process have not been completely elucidated, it is known that it involves a diverse number of enzymatic activities. Among these, it is well established that proteins related to carbohydrate metabolism, such as callose synthases or expansins, have an important role in wall loosening and extension during pollen germination [3,4]. The pollen tube wall displays a characteristic primary wall composed largely of pectin, hemicellulose and cellulose, and a secondary callosic wall [2]. The pollen tube grows exclusively at the tip, which contains more methyl-esterified pectin than other wall regions distal to the tip wall [5–8]. Pollen germination also involves the secretion of proteins from the pollen grain or the pollen tube. In fact, imbibed pollen releases proteins present in the coat, in the wall, or secreted from the pollen interior. Some of these proteins have been described to induce type I allergy in humans [9].

Olive tree (*Olea europaea*) pollen is one of the main causes of allergy in Mediterranean countries, where this tree is widely cultivated. To date, ten allergens, named Ole e 1 to Ole e 10, have been isolated and characterized from this pollen [10–12]. Ole e 10, a small (10.8 kDa) and acidic (pI 5.8) protein, has been recently described as a major allergen from this pollen, since it affects more than 55 % of allergic patients [12]. The allergen was shown to be implicated in IgE cross-reactivity among pollens, fruits and latex, suggesting the ubiquitous presence of Ole e 10-like proteins in different sources. Furthermore, the clinical relevance of Ole e 10 has been recently enhanced by the description of an association between asthma and sensitivity to Ole e 10 found in olive pollen-allergic patients, pointing to a possible role of this allergen in the development and exacerbation of asthmatic processes [13].

Ole e 10 shows identity with amino acid sequences deduced from *Arabidopsis thaliana* genes, but protein products derived from the expression of these genes have not yet been reported, and their putative biological functions are unknown. Ole e 10 also shows similarity with the C-terminal domain (approx. 100 amino acid residues) of long 1,3-β-glucanases, which also have an N-terminal domain (approx. 300 amino acids in length) containing the catalytic site [11,14]. The C-terminal domain, the biological role of which is unknown, is absent from most 1,3-β-glucanases described to date [15]. Finally, Ole e 10 shows similarity with polypeptide segments – named Cys boxes – of 1,3-β-glucanosyltransferases involved in yeast development: Epd (essential for pseudohyphal development) [16], Gas (glycophospholipid-anchored surface) [17–19] and Phr (pH-regulated) [20]. However, unlike these modules of glucanases and glucanosyltransferases, Ole e 10 is an independent protein that is not covalently attached to another polypeptide domain.

Enzymes that are implicated in carbohydrate metabolism usually consist of a catalytic domain plus one or several non-catalytic CBMs (carbohydrate-binding modules), which may enhance

Abbreviations used: AGE, affinity gel electrophoresis; bM_w, buoyant molar mass; CBM, carbohydrate-binding module; CLSM, confocal laser scanning microscopy; Epd, essential for pseudohyphal development; Gas, glycophospholipid-anchored surface; Phr, pH-regulated; TEM, transmission electron microscopy.

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enzymatic activity by improving access to the substrate [21]. Although the first CBM definitions referred to non-catalytic sugar-binding domains from glycosyl hydrolases, this term also includes sugar-binding domains from structural proteins and some independent putative CBMs that are not directly appended to enzymatically active modules [21]. Based on similarities in primary structure, CBMs have been classified into 42 different families and grouped in the CAZy database (http://afmb.cnrs-mrs.fr/CAZY).

In the present study, we analyse the biochemical activity of Ole e 10 by means of chemical, physical and spectroscopic experiments, as well as its cellular location by TEM (transmission electron microscopy) and CLSM (confocal laser scanning microscopy). The protein can be described as an independent CBM not linked to another polypeptide module, and represents the first member of a new CBM family, for which we propose the name CBM43.

EXPERIMENTAL

Materials

Ole e 10 was isolated from olive pollen as described previously [12]. Laminarin (1,3-β-glucan, from *Laminaria digitata*), lichenan (1,3/1,4-β-glucan, from *Cetraria islandica*) and CM-celullose (1,4-β-glucan) were purchased from Sigma. Agarose (1,3-β/1,4-α-galactose) was purchased from Conda Labs. Laminarihexaose [(1,3-β-glucan)$_6$] was purchased from Megazyme International.

Laminarin was subjected to MS analysis, for which the sample (5 μg) was mixed with a matrix solution composed of saturated α-cyano-4-hydroxycinnamic acid in 30 % (v/v) aqueous acetonitrile and 0.1 % (v/v) trifluoroacetic acid. Measurements were performed in a Bruker Reflex II matrix-assisted laser desorption ionization time-of-flight mass spectrometer (Bruker-Franzen Analytik) equipped with an ion source with visualization optics and a nitrogen laser (337 nm). The equipment was externally calibrated employing singly, doubly and triply charged signals from either cytochrome *c* (12360 Da) or BSA (66430 Da). Chains from 23 to 27 glucose units were identified as the major polysaccharide components of laminarin, although other minor constituents with lower and higher molecular masses were also present.

AGE (affinity gel electrophoresis)

The capacity of Ole e 10 to bind soluble polysaccharides was evaluated by AGE as described previously [22] with minor modifications. Briefly, native polyacrylamide gels were prepared, consisting of 15 % (w/v) acrylamide in 0.3 M Tris buffer, pH 8.8. Different amounts of saccharides (0.1–5 mg/ml) were added to the separating gel mixtures prior to polymerization. Ole e 10 (2 μg) was electrophoresed at 25 mA/gel for 1 h at room temperature. Gels without ligand were run simultaneously under the same conditions. BSA (0.7 μg; from Sigma) was used as a negative non-interacting control. Proteins were visualized by staining with Coomassie Blue R-250. The migration distances of both Ole e 10 and BSA were measured from the top of the gel, and these data were used to determine the dissociation constant (K_D) from plots of $1/(R_0 - r)$ against $1/C$ according to the affinity equation:

$$1/(R_0 - r) = 1/(R_0 - R_C)(1 + K_D/C) \quad (1)$$

where r is the relative migration distance of Ole e 10 in the gel in the presence of ligand, R_0 is the relative migration distance of free Ole e 10 in the absence of ligand, R_C is the relative migration distance of the complex at a high excess of ligand where all Ole e 10 molecules are fully complexed, C is the concentration of the ligand in the gel, and K_D is the dissociation constant of Ole e 10 for the ligand. K_D values were determined as the inverse of the absolute value of the intercept on the abscissa of data plotted according to the affinity equation. All migration distances of Ole e 10 were measured relative to the migration of the reference protein BSA.

Analytical ultracentrifugation analysis

The state of association of Ole e 10 alone (5.8 μM) and in the presence of laminarin (1.23, 2.45 and 6.00 mM in glucose-equivalents units) was characterized by means of short-column (75 μl) sedimentation equilibrium. The experiments were performed in 20 mM sodium phosphate buffer, pH 7.2, at 25 °C and 50000 ***g*** in an Optima XL-A analytical ultracentrifuge (Beckman-Coulter Inc.), equipped with absorbance optics, using an An-60Ti rotor and standard (12 mm optical path) double-sector centrepieces of Epon-charcoal. Absorbance scans at equilibrium were carried out at wavelengths at which the absorption of the sugar was negligible, and therefore only the protein gradient was monitored. Baseline offsets were measured afterwards at 200000 ***g***.

Whole-cell weight-average bM_w (buoyant molar mass) values were obtained by fitting the experimental data to the equation for the radial concentration distribution of an ideal solute at sedimentation equilibrium, using the program EQASSOC supplied by Beckman-Coulter [23]. Analysis of the sedimentation equilibrium data for the mixtures of Ole e 10 with laminarin was performed assuming the linear approximation for the buoyant masses [24]:

$$\mathrm{b}M_{w,C} = i\,\mathrm{b}M_{w,P} + j\,\mathrm{b}M_{w,L} \quad (2)$$

where b$M_{w,P}$ and b$M_{w,L}$ are the bM_w values for pure Ole e 10 (denoted by P) and pure laminarin (denoted by L) respectively, and C refers to the complex P_iB_j formed by i molecules of Ole e 10 and j molecules of laminarin (expressed as the equivalent number of molecules of glucose). The corresponding apparent weight-average molar masses ($\overline{M}_w$) were determined from the buoyant masses, taking into account the partial specific volumes of the protein (0.704 ml/g; obtained from the amino acid composition using the program SEDNTERP [25]) and of laminarin (because it is a 1,3-β-glucose polymer, we have used the value of 0.622 ml/g reported for glucose by Perkins et al. [26]).

CD analyses

CD spectra were obtained using a Jasco J-715 spectropolarimeter (Japan Spectroscopic Co.) fitted with a 150 W xenon lamp and connected to a Neslab RTE-111 thermostabilizer bath. Far-UV spectra of Ole e 10 (0.2 mg/ml), in absence or presence of laminarin (1 mg/ml), were registered in the range 190–250 nm using optical-path cell of 0.1 cm. Samples were analysed in 20 mM sodium phosphate, pH 7.2, at 25 °C. Mean residue mass ellipticities were calculated based on 106 Da as the average molecular mass/residue, obtained from the amino acid composition, and expressed in terms of θ ($\mathrm{deg \cdot cm^2 \cdot dmol^{-1}}$). Final spectra were corrected by subtracting the corresponding baseline spectrum obtained for the buffer alone under identical conditions. Secondary structure estimations were performed by computer fit according to the method of Perczel et al. [27].

The temperature-induced denaturation of Ole e 10, in the absence or presence of laminarin (1 mg/ml), was monitored by CD measurements at 220 nm. The temperature was increased from 20 to 80 °C at 0.5 °C/min. Denaturation data are represented as the percentage denaturation of Ole e 10 in the absence and presence of laminarin at each temperature. These values were calculated by

considering the θ values at 20 and 80 °C as representing 0 % and 100 % denaturation respectively.

Denaturation curves were analysed using the two-state model. The difference in free energy (Gibbs energy) between the native and the denatured states, ΔG, was calculated using the equation:

$$\Delta G = -RT \ln K \quad (3)$$

where R is the gas constant (8.31 J/K per mol), T is the absolute temperature and K is the equilibrium constant. The corresponding thermodynamic parameters, T_m (the midpoint of the thermal denaturation curve), ΔH_m (the enthalpy change at T_m) and ΔS_m (the entropy change at T_m), were determined from plots of ΔG against T. In the midpoint of a thermal denaturation curve:

$$\Delta G = 0 = \Delta H_m - T_m \Delta S_m \quad (4)$$

This gives ΔS_m as the slope of the plot of ΔG against T at T_m, and $\Delta H_m = T_m \Delta S_m$. The difference in the free energy change associated with the carbohydrate binding, $\Delta(\Delta G)$, was calculated as:

$$\Delta(\Delta G) = \Delta(T_m)\Delta S_m = \Delta T_m(\Delta H_m / T_m) \quad (5)$$

where $\Delta(T_m)$ is the difference between the ΔT_m values measured for Ole e 10 in the presence and absence of carbohydrate, whereas ΔS_m and ΔH_m are values for the carbohydrate-free forms of the protein [28].

Fluorescence analysis

Fluorescence emission spectra were obtained using an SLM Aminco 8000 spectrofluorimeter at 25 °C in a 0.2 cm optical-path cell, using 4 nm slits for both excitation and emission beams. The protein concentration was 4.6 μM in Tris buffer, pH 8.8. Different amounts of laminarin (0, 3.6, 10.9, 18.2, 36.3, 72.6, 181.6, 290.5, 363.1 and 544.7 μM) or laminarihexaose (0, 10.2, 30.8, 51.4, 102.9, 205.7 and 514.4 μM) were added. Final spectra were corrected for protein concentration, and the corresponding baseline spectrum performed in the presence of the corresponding amount of carbohydrate was subtracted. The molar concentration of laminarin was calculated using a value of 17 glucose units as the average Ole e 10-carbohydrate bound structure, as determined from the ultracentrifugation analyses.

The titration curve for Ole e 10–carbohydrate binding was represented as the increase in fluorescence at 336 nm (ΔF_{336}), calculated from the emission spectra of Ole e 10 in the presence and absence of the sugar, with respect to the concentration of carbohydrate added to the sample. The data were analysed by non-linear regression using a standard single-site binding model, and the K_D value was calculated from the regressed data.

Pollen germination *in vitro*

Mature pollen grains were collected from selected *O. europaea* L. trees (cv. Picual) in Granada (Spain) during the anthesis period. Pollen was sieved through meshes of pore sizes 150 and 50 μm in order to separate pollen grains from debris. Grains were then frozen in liquid nitrogen and stored at −80 °C. Stored pollen was pre-hydrated by incubation in a humid chamber at room temperature for 30 min and then transferred to Petri dishes (0.1 g/plate) containing 10 ml of germination medium [10 % (w/v) sucrose, 0.03 % (w/v) $Ca(NO_3)_2$, 0.01 % (w/v) KNO_3, 0.02 % (w/v) $MgSO_4$ and 0.01 % (w/v) boric acid]. Petri dishes were maintained at room temperature in the dark, and pollen samples were taken 8 h after the onset of the culture by transferring the culture to 1.5 ml disposable plastic tubes.

Immunolocalization of Ole e 10 by TEM and CLSM

For TEM studies, samples were processed as reported by Alché et al. [29], with minor modifications. Mature pollen grains were fixed for 4 h at 4 °C with 4 % (w/v) paraformaldehyde and 0.2 % (w/v) glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2. Samples were dehydrated in an ethanol series, gradually transferred to propylene oxide and embedded in Unicryl resin (BBInternational). Ultrathin sections (80 nm) were obtained using a Reichert–Jung ultramicrotome and then transferred on to formvar-coated 300-mesh nickel grids. Blocking of non-specific binding sites was carried out by incubation of sections for 15 min in a blocking solution containing 5 % (w/v) BSA in PBS. Blocking was followed by incubation at room temperature for 2.5 h with a monoclonal antibody against Ole e 10, diluted 1:20 in blocking solution. After washing with PBS, the grids were treated for 2 h with a goat anti-(mouse IgG) secondary antibody coupled to 20 nm gold particles (BBInternational), diluted 1:30 in PBS. Finally, they were washed in PBS, rinsed in double-distilled water, and then stained for 10 min with 5 % (w/v) uranyl acetate. Observations were carried out using a Zeiss EM10C transmission electron microscope. Treatment of control sections was the same, except that incubation with the primary antibody was omitted.

For CLSM studies, *in vitro*-germinated pollen grains were separated from the culture medium by centrifugation (5000 ***g*** for 10 min) and fixed at 4 °C for 30 min in a solution containing 4 % (w/v) paraformaldehyde in washing buffer A (50 mM Tris/HCl, pH 6.8). After washing in buffer A, the samples were subjected to a permeation procedure by incubation in pre-cooled (−20 °C) acetone for 10 min at −20 °C and further washing with buffer A. Samples were subsequently incubated at 4 °C in the following solutions, using gentle shaking: blocking solution [5 % (w/v) BSA, 1 % (v/v) Triton X-100 prepared in washing buffer] for 10 min; washing solution B [1 % (v/v) Triton X-100 in washing buffer A], three changes of 10 min each; anti-Ole e 10 polyclonal antibody (diluted 1:50 in blocking solution) overnight; washing solution B as above; goat anti-(rabbit IgG) Cy3-conjugated secondary antibody (Sigma), diluted 1:500 in blocking solution, 2 h in the dark; and final rinses in washing solution B. Double-labelling experiments with Ole e 10 and callose were carried out by additionally incubating the samples for 30 min with 0.025 mg/ml sirofluor (Biosupplies) in distilled water after the immunolocalization protocol. Finally, samples were re-suspended in a CITIFLUOR/glycerol/PBS antifading solution (Sigma) and observed in a Nikon C1 confocal laser scanning microscope using a He/Ne laser (543 nm) for immunofluorescence (plus an Ar laser at 488 nm for double-labelling experiments). Negative controls were treated in the same way, except that the primary antibody was omitted.

RESULTS

Binding experiments using AGE

AGE with non-denaturing gels was used to analyse the ability of Ole e 10 to bind specifically to soluble polysaccharides (Figure 1). The analysis revealed an interaction between Ole e 10 and laminarin, and a slight retardation in the presence of CM-cellulose. No binding to agarose or lichenan was detected, indicating a preferential interaction between Ole e 10 and 1,3-β-glucans.

Quantitative binding of Ole e 10 to soluble polysaccharides was evaluated by AGE in the presence of various amounts of laminarin (Figure 2). The reciprocal relative migration distance $1/(R_0 - r)$ was plotted against the carbohydrate concentration. The K_D for the binding of Ole e 10 to the ligand under the conditions described

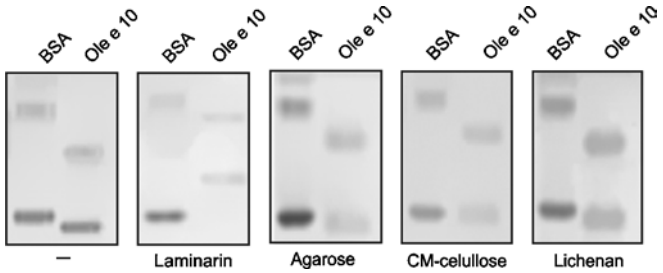

Figure 1 Qualitative AGE analysis of the interaction of Ole e 10 with soluble polysaccharides

Ole e 10 (2 μg) and BSA (0.7 μg) were electrophoresed on non-denaturing polyacrylamide gels containing polysaccharide (1 mg/ml) or no polysaccharide (—).

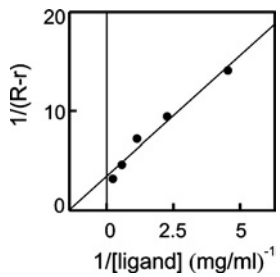

Figure 2 Quantitative AGE with laminarin as ligand

Ole e 10 and BSA were subjected to non-denaturing gel electrophoresis on gels containing different concentrations of laminarin. The data obtained were plotted using the equation described in the Experimental section.

was determined as the inverse of the absolute value of the intersect of the plot with the abscissa. According to this method, the K_D for the binding to laminarin was determined to be 0.79 mg/ml.

Analytical ultracentrifugation

Figure 3 plots the sedimentation equilibrium gradients of Ole e 10 (5.8 μM) alone and in the presence of laminarin (2.45 mM in glucose-equivalent units), and shows the effect of the sugar on protein behaviour. In the absence of laminarin, Ole e 10 sedimented at equilibrium as a single species with a best-fit bM_w of 3300 ± 100 (Figure 3, open circles) which, after buoyancy correction, is compatible with the expected molar mass of the Ole e 10 monomer on the basis of its amino acid composition (10 785 Da). Addition of laminarin [b$M_w = 1545$, calculated from the molar mass of the major component of the sugar (4094 Da) determined by MS] to the Ole e 10 solution modified the sedimentation behaviour of the protein: the gradient was steeper than for the protein alone, corresponding to a bM_w of approx. 4400 ± 100 (Figure 3). This value is compatible with the formation of a complex between one Ole e 10 molecule and approx. 17 molecules of glucose. From these results, assuming that Ole e 10 binds laminarin molecules with 17 glucose units, the K_D value for the binding of Ole e 10 to laminarin obtained by AGE would be 290 μM.

Figure 4 summarizes the effect of laminarin concentration upon the binding of the sugar to the Ole e 10 protein. At the lowest concentration of laminarin added to the protein (1.23 mM glucose-equivalent units) the amount of sugar bound to Ole e 10 was approx. 15 glucose units, a value that changed only slightly with increasing amounts of laminarin, reaching 18 ± 1 glucose units at the highest concentration of sugar used (6 mM).

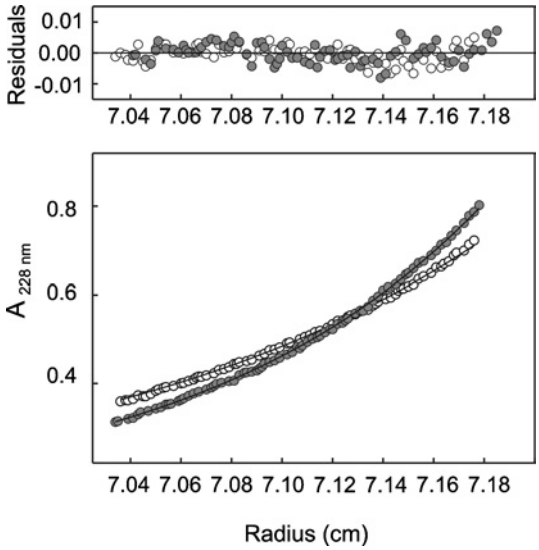

Figure 3 Analytical ultracentrifugation analysis

Sedimentation equilibrium gradients (50 000 ***g***, 25 °C) are shown for Ole e 10 alone (○) and in the presence of laminarin (grey circles). The solid lines show the corresponding best-fit gradients for a single sedimenting species at sedimentation equilibrium (with bM_w values for the protein and the protein–laminarin complex of 3300 ± 100 and 4400 ± 100 respectively). The concentrations of protein and laminarin were 5.8 μM and 2.8 mM in equivalent glucose units respectively.

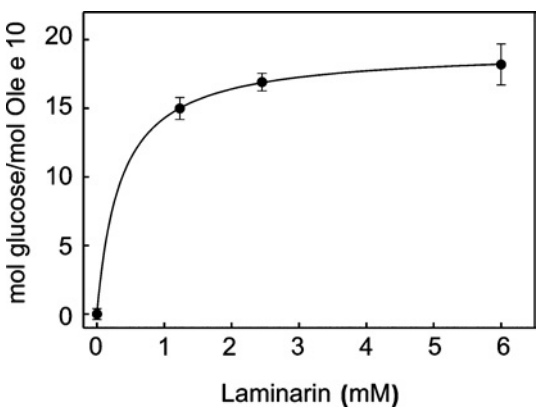

Figure 4 Binding of laminarin to Ole e 10 as assessed by sedimentation equilibrium

The amount of sugar bound to the protein was calculated from the change in the bM_w of Ole e 10 upon the addition of laminarin, as described in the Experimental section. The solid line is drawn to show the trend of the data.

Spectroscopic analysis

The content of regular elements of secondary structure in Ole e 10 was determined by obtaining its far-UV CD spectrum in the absence and in the presence of laminarin (Figure 5A). Laminarin induced a modification in the shape of the spectra of Ole e 10 and in the molar ellipticity values, indicating a change in the secondary structure of the protein. Application of the convex-constraint-analysis method of Perczel et al. [27] to these spectra allowed the quantification of this change in secondary structure composition (Table 1). Thermal denaturation curves for Ole e 10 in the presence or absence of laminarin (Figures 5B and 5C) revealed that the interaction with the carbohydrate increased the stability of Ole e 10, since the T_m value determined in the presence of laminarin (61.2 °C) was higher than that obtained in the

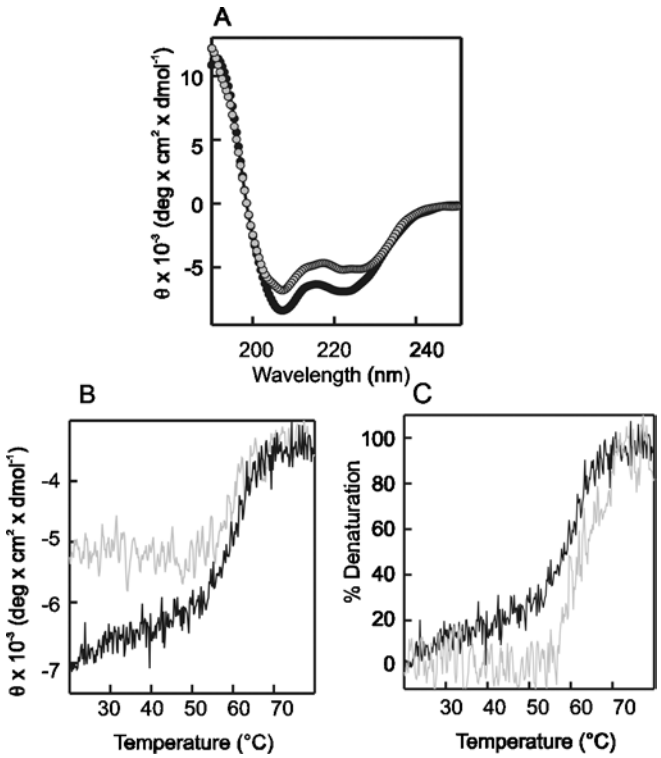

Figure 5 CD analyses of Ole e 10 in the presence of laminarin

(**A**) Far-UV (190–250 nm) CD spectra of Ole e 10 in absence (black circles) or presence (grey circles) of laminarin. Temperature-induced denaturation of Ole e 10 in the absence (black line) or presence (grey line) of laminarin was monitored by CD measurements at 220 nm (**B**) and expressed as percentage denaturation (**C**).

Table 1 Secondary structure composition of Ole e 10 determined from CD spectra

	Secondary structure content of Ole e 10 (%)	
	Ole e 10 alone	Ole e 10 + laminarin
α-Helix	17	15
β-Sheet	33	37
Turn	21	21
Coil	29	27

absence of the glucan (57.6 °C). The application of the two-state model to the denaturation process allowed us to determine a change in ΔG of 2.1 kJ/mol for the interaction of Ole e 10 with laminarin (Table 2).

To analyse the influence of carbohydrate binding on the tertiary structure of Ole e 10, fluorescence emission spectra of the protein in the absence and presence of different amounts of laminarin were recorded (Figure 6). Changes in the surroundings of the two Trp residues of Ole e 10 [12] were analysed by measurement of the fluorescence intensity values after excitation of the samples at 295 nm (Figure 6). Ole e 10 fluorescence emission spectra in the absence of laminarin showed two maxima (around 328 and 336 nm), indicating a different polarity in the local environment of both Trp residues. The addition of increasing amounts of laminarin induced a modification in the shape of the plot and a decrease in the fluorescence ratio at the maximum emission value for both Trp residues (F_{336}/F_{328}) (Figure 6, inset). This could indicate that the Ole e 10–carbohydrate interaction affects the two

Table 2 Energetic parameters calculated from the denaturation curves of Ole e 10 in the presence or absence of laminarin

ΔS_m is the slope of the plot of ΔG against T at T_m. ΔH_m is given by $\Delta H_m = T_m(K)\Delta S_m$. T_m represents the midpoint of the thermal denaturation curve. $\Delta(T_m)$ is the difference between the T_m values measured for the protein with and without carbohydrate. $\Delta(\Delta G)$ is given by $\Delta(\Delta G) = \Delta(T_m)\Delta S_m = \Delta(T_m)(\Delta H_m/T_m)$, where ΔS_m and ΔH_m are values for the forms of the protein without carbohydrate.

Parameter	Ole e 10 alone	Ole e 10 + laminarin
ΔS_m (J/mol per K)	534.5	996.7
ΔH_m (kJ/mol)	176.6	333.2
T_m (°C)	57.6	61.2
$\Delta(T_m)$ (°C)	3.6	3.6
$\Delta(\Delta G)$ (kJ/mol)	2.1	2.1

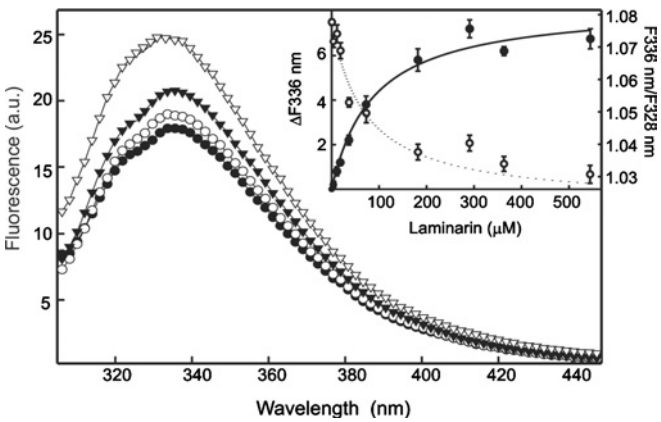

Figure 6 Fluorescence analysis of Ole e 10 in the presence of laminarin

Shown are fluorescence emission spectra (300–450 nm) of Ole e 10 after excitation at 295 nm, in the presence of different amounts of laminarin (μM): 0 (●), 10.9 (○), 18.2 (▼) and 72.6 (▽). Inset: increase in fluorescence intensity (●) at 336 nm determined from Ole e 10 emission spectra against laminarin concentration; the fluorescence intensity ratio (○) at the maximum emission value for both Trp residues (F_{336}/F_{328}) is also shown. Carbohydrate concentrations were calculated assuming that Ole e 10 binds laminarin molecules with 17 glucose units.

Trp residues in different ways. The variation of the fluorescence intensity at 336 nm dependent on the laminarin concentration (Figure 6, inset) showed a saturating behaviour. From these results, assuming that Ole e 10 binds laminarin molecules with 17 glucose units, the estimated K_D for the Ole e 10–laminarin interaction in soluble conditions would be 88 μM. Fluorescence emission spectra of Ole e 10 in the presence of different amounts of laminarihexaose were also obtained, but no differences in the spectra were detected.

Immunolocalization of Ole e 10 in mature and germinating olive pollen

In the mature pollen grain (Figure 7A), Ole e 10 epitopes were localized in the cytoplasm of the vegetative cell. Gold particles were observed mainly inside Golgi-derived vesicles. The pollen cell walls (intine and exine), the generative cell and both nuclei were practically free of gold particles. No gold labelling was found in control sections prepared by omitting the primary antibody (Figure 7B).

Figure 8 illustrates the double localization of Ole e 10 and callose by CLSM. Labelling of callose in the pollen tube by the fluorescent probe Sirofluor appeared as a greenish fluorescence occurring mainly in the tube plugs (Figure 8) and the thin innermost layer of the pollen tube wall, adjacent to the plasmalemma.

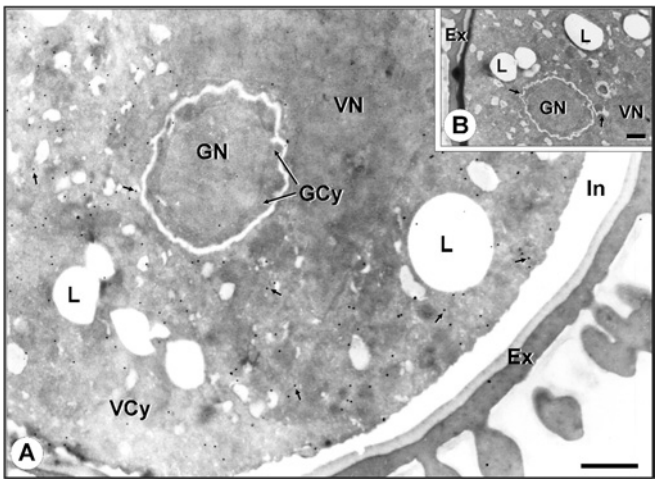

Figure 7 TEM immunolocalization of Ole e 10 in the mature olive pollen grain

(**A**) Gold particles are localized inside Golgi-derived vesicles in the cytoplasm of the vegetative cell (unlabelled arrows). The pollen cell walls, the generative cell and both nuclei are devoid of labelling. (**B**) Control section prepared by omitting the primary antibody. Only a few particles are present. Ex, exine; GCy, generative cell cytoplasm; GN, generative cell nucleus; In, intine; L, lipids; VCy, vegetative cell cytoplasm; VN, vegetative cell nucleus. Bars represent 1 μm.

Callose plugs were present in the pollen tube at intervals, and ranged in number between one and four. Immunofluorescence labelling of Ole e 10 became visible as red fluorescence located in the outermost layer of the pollen tube wall, frequently forming dense red spots (Figure 8C). The cytoplasm of the pollen tube also displayed red fluorescence, which co-localized with callose and therefore appeared as yellowish fluorescence. Negative control sections (not shown) did not exhibit significant fluorescence above background levels.

DISCUSSION

Ole e 10 has been described recently as a major olive pollen allergen, and represents the first isolated and characterized member of a new family of plant proteins whose biochemical activity was previously unknown [12]. In the present study, the ability of Ole e 10 to bind soluble polysaccharides has been demonstrated. In the absence of similar CBMs reported to date, Ole e 10 can be described as the first member of a novel family of CBMs, for which we propose the name CBM43, according to the classification of the CAZy database. Ole e 10 binds specifically 1,3-β-glucans, with K_D values for the Ole e 10–laminarin interaction of 290 μM (as assessed by AGE) and 88 μM (by fluorescence). The monomeric state of Ole e 10 in its binding to laminarin was confirmed by analytical ultracentrifugation, which demonstrated a 1:1 stoichiometry for the Ole e 10–carbohydrate interaction.

Although most CBMs described to date exist as modules of larger enzymes, some non-catalytic carbohydrate-binding proteins contain free CBMs. This is the case for cellulosomal scaffolding proteins (CBM3), several independent putative CBMs (family 18), and NCP1 protein from the anaerobic fungus *Piromyces equi* (CBM29) [30]. These CBMs essentially act as scaffolding proteins, being present in multi-enzyme complexes implicated in carbohydrate metabolism, such as the cellulase–hemicellulase complex, in which the CBMs may mediate carbohydrate attachment [30]. In this context, Ole e 10 could act as a CBM to regulate the activity of different enzymes. On the other hand, Ole e 10 shows sequence identity with the non-catalytic C-terminal do-

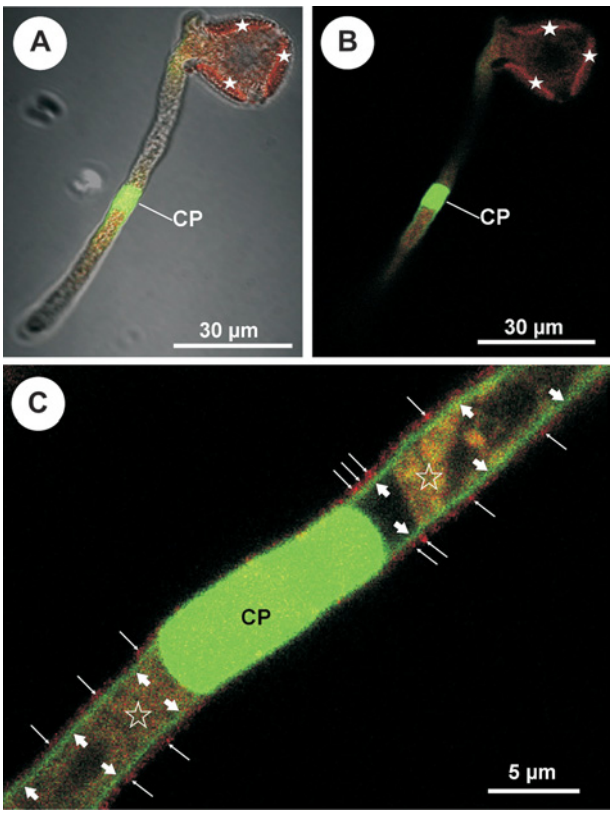

Figure 8 CLSM co-localization of Ole e 10 and callose materials in olive pollen grains germinating *in vitro*

(**A**, **B**) Germinating olive pollen grain showing a callose plug (CP) observed by differential interferential contrast plus CLSM (**A**) and CLSM only (**B**). Optical sections show greenish fluorescence localized in the CP and the innermost layer of the pollen tube wall. Red autofluorescence can be observed in the pollen exine (filled stars). (**C**) High-magnification CLSM optical section of a pollen tube with a CP. Both the CP and the innermost layer of the pollen tube wall (thick short arrows) are intensely stained by Sirofluor. Ole e 10 is located in the outermost layer of the pollen tube wall, frequently forming dense red spots (thin long arrows), and in the pollen tube cytoplasm, co-localizing with callose and therefore appearing as yellowish fluorescence (empty stars)

mains of plant 1,3-β-glucanases [11,14,15] (27–53 % identity, 44–69 % similarity) and with Cys-box domains from yeast glucanosyltransferases [16–20] (Gas, Epd and Phr families; 23 % identity, 40–43 % similarity) of unknown biological or biochemical functions [12]. This similarity suggests a similar functional role for the two polypeptides in acting as CBMs facilitating ligand accessibility to the catalytic site, with Ole e 10-like proteins operating as free molecules (e.g. as a subunit of polymeric protein complexes) and Cys-box domains of glucanases and glucanosyltransferases fulfilling the same function as covalently bound domains. Finally, based on their identity with Ole e 10 [12], these Ole e 10-like domains could be included in this new CBM43 family.

The structural changes that occur in Ole e 10 during its interaction with carbohydrates have been studied by employing spectroscopic analyses. CD experiments demonstrated a slight but significant change in the secondary structure of Ole e 10 in the presence of laminarin. This change is in agreement with the fact that CBMs appear to have pre-formed carbohydrate recognition sites that mirror the solution conformations of their target sugars [21]. In addition, fluorescence studies showed a change in the environment of aromatic residues of Ole e 10, as a consequence of the interaction with the carbohydrate. Boraston et al. [21] have

recently reported that the side chains of tryptophan, tyrosine and, less commonly, phenylalanine residues usually form the hydrophobic platforms in CBM binding sites. As an example, a conserved core with three aromatic residues has been implicated in protein–carbohydrate interactions of Cel9B from *Cellulomonas fimi* and Lam16A from *Thermotoga maritime* (family 4) [31]. On the other hand, most of the aromatic residues of Ole e 10 are highly conserved in the similar domains in plant 1,3-β-glucanases and yeast glucanosyltransferases [12]. This conservation in different proteins and different organisms supports a role in carbohydrate binding.

The biochemical activity of Ole e 10 established at this point may also help to explain certain aspects of its biological role in the pollen grain. The involvement of carbohydrates in pollen germination and pollen tube growth is well known. Among these, callose (1,3-β-glucan), one of the major components of the pollen tube wall, is deposited behind the tube tip as an inner-wall layer, and also forms the plugs that traverse older regions of the pollen tube. Localization of Ole e 10 within the mature pollen grain (inside Golgi-derived secretory vesicles) and its co-localization with callose materials in the pollen tube cytoplasm indicate the presence of dynamic changes in the localization of the protein, which is most probably secreted to the pollen tube wall upon pollen germination and pollen tube growth. Although the mechanisms underlying carbohydrate metabolism during pollen tube growth are not completely clear, a diverse number of enzymatic activities must be implicated in the synthesis and degradation of components of the pollen wall. At this level, the involvement of a callose synthase activity during pollen tube growth is well established [3,32], but less is known about the possible role of degradative enzymes such as 1,3-β-glucanases. Detailed analyses of the expression of 1,3-β-glucanases have indicated that these proteins are not normally expressed in the microspore or the pollen interior, but in the anther [9,33]. However, the expression of two exo-β-glucanases (LP-exo I and LP-exo II) in both the pollen grain and the pollen tube of the lily (*Lilium longiflorum*) has been reported recently [34]. These glucanases are secreted into the cell walls of the lily pollen tube, and a possible role in the regulation of pollen tube elongation, via hydrolysis of callose and 1,3:1,4-β-glucan within the pollen tube walls, has been suggested. Our results regarding the immunolocalization of Ole e 10 in the outermost layer of the pollen tube wall, close to the adjacent innermost callose wall, suggest the participation of the protein in this kind of process. Thus Ole e 10 could act as a carbohydrate-binding protein that interacts with 1,3-β-glucans and regulates the enzymatic activity of proteins implicated in cell wall synthesis/degradation during pollen germination, as a part of a multi-protein complex similar to that described for the cellulase–hemicellulase complex.

Finally, elucidation of the biochemical activity of Ole e 10 and its localization in the pollen grain could help to explain the immunological behaviour of the protein. Although Ole e 10 has been described as a prevalent allergen, preliminary observations reported a low intrinsic antigenicity for this molecule [12]. This fact, added to the retardation in the release of Ole e 10 from the pollen cell after hydration – although the protein is highly soluble *per se* – were interpreted on the basis that the allergen may be expelled from the pollen linked to a particle that behaves as a carrier, thus increasing its potential antigenicity [12]. In this sense, several studies have shown an adjuvant activity of carbohydrates in the development of the allergic response [35,36]. These authors described the role of different kinds of particles (mainly starch granules) that act as carriers for grass pollen allergens and facilitate their access to the respiratory tract. However, this mechanism would not be applicable to olive pollen, as ultrastructural studies carried out on this pollen [37] have determined that it does not display significant amounts of starch granules. Nevertheless, the biochemical activity of Ole e 10, its co-localization with carbohydrate particles in the pollen, as well as the effects of the inhalation of 1,3-β-glucans reported in humans (in which these carbohydrates promote airway allergic responses [38] and cause respiratory symptoms [39]), support the hypothesis of a interaction of Ole e 10 with some type of carbohydrate carrier such as 1,3-β-glucan polymers that could increase its antigenic potential and contribute to eliciting asthmatic reactions in allergic patients [13].

In conclusion, Ole e 10 defines a new family of CBMs, for which we suggest the name CBM43. The sequence identity described between Ole e 10 and the non-catalytic domains of 1,3-β-glucanases and glucanosyltransferases supports the inclusion of these domains into this CBM family, although their carbohydrate-binding capabilities must be demonstrated.

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