Ole e 9, a Major Olive Pollen Allergen Is a 1,3-β-Glucanase
ISOLATION, CHARACTERIZATION, AMINO ACID SEQUENCE, AND TISSUE SPECIFICITY*

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Olive pollen allergy is a clinical disorder affecting the human population of Mediterranean areas. A novel major allergen, Ole e 9, has been isolated from olive pollen by gel permeation, hydrophobic affinity, and reverse-phase high performance liquid chromatographies. It is involved in the allergic responses of 65% of patients suffering olive pollinosis. Ole e 9 (molecular mass of 46.4 kDa) displays 1,3-β-endoglucanase activity (38.9 ± 5.6 mg of glucose released/min × μmol of protein at pH 4.5–6.0 using laminarin as substrate). It is the first 1,3-β-glucanase, a member of the “pathogenesis-related” protein family, detected in pollen tissue. Seven tryptic peptides of the allergen were sequenced by Edman degradation and used for designing primers to clone the cDNA coding the protein. Specific cDNA for Ole e 9 was synthesized from total RNA and amplified using the polymerase chain reaction. The allergen sequence showed an open reading frame of 460 amino acids comprising a putative signal peptide of 26 residues. It shows 39, 33, and 32% sequence identity including the catalytic residues when compared with 1,3-β-glucanases from wheat, willow, and Arabidopsis thaliana, respectively. Northern blot analysis showed that Ole e 9 transcript is specifically expressed in the pollen tissue, and highly conserved counterparts were only detected in taxonomically related pollens.

Identification, isolation, and characterization of proteins responsible for IgE-mediated allergies have been the main goals of research in the last few years, because type I allergy is being an increasing clinical disorder for the human population. Accurate diagnosis and efficient immunotherapy protocols are strongly dependent on the use of standardized extracts or well characterized allergen mixtures (1). Unfortunately, allergenic sources frequently contain complex mixtures of allergens from which only a limited number is currently well known.

Olive pollinosis is one of the most important causes of inhalant allergy in Mediterranean countries and some areas of North America (2–4). Previous studies have reported the presence of a high number of allergens in olive (Olea europaea) pollen extracts, at least 20 fractions with allergenic activity using IEF1, radioallergosorbent test, and radioallergosorbent test inhibition (5). Eight olive allergens, Ole e 1–8, with different incidences among the allergic patients so far have been completely or partially characterized (6–13). All these proteins have molecular masses of <35 kDa (Ole e 1 = 20 kDa, profilin Ole e 2 = 16 kDa, polcalcin Ole e 3 = 9.2 kDa, Ole e 4 = 32 kDa, Ole e 5 = 16 kDa, Ole e 6 = 5.8 kDa, Ole e 7 = 10 kDa, and Ole e 8 = 18.8 kDa). But the allergenic pattern of olive also displays polypeptides of higher molecular mass that have not been explored yet. IgE reactive proteins of 42, 45–47, 60–65, and 70 kDa have been detected in the pollen extract (12, 14–17), and some of them seem likely to be of high clinical significance close to that of Ole e 1, the main allergen from olive pollen, to which >70% olive allergic patients are sensitive as deduced from the prevalence observed in immunoblotting assays (6, 15, 16).

Some allergens have been biochemically characterized. Thus, cysteine-protease, hyaluronidase, or glutathione S-transferase are enzyme activities observed for Der p 1, hymenoptera venom allergens, and Bla g 5, respectively (18–20). Among pollen allergens, profilins (Bet v 2), defense-related proteins (group 1 allergens such as Bet v 1, Aln g 1, and Cor a 1), Ca2+-binding proteins (Bet v 3, Bet v 4, Cyn d 7, Bra n 1, Phl p 7, and Aln g 4), or pectate lyase (Cry j 1) have been described previously (21–24). Olive pollen extracts contain profilin (Ole e 2), two allergens belonging to the EF-hand Ca2+-binding protein family (Ole e 3 and Ole e 8), and superoxide dismutase (Ole e 5) (8–10, 13). In addition, Ole e 1 has been suggested to be involved in the hydration of the pollen during germination (6). The biological function of Ole e 4, Ole e 6, and Ole e 7 cannot be deduced because neither biochemical activity has been demonstrated nor were putative homologous polypeptides found in EMBL/GeneBank/DDBJ databases. The knowledge of the biochemical role of a novel allergen can substantially improve the strategy for its purification as well as its analysis and genetic manipulation, and it can also help to explain relationships between the biological function and allergenic activity (25, 26). Moreover, the analysis of the similarity among homologous counterparts from different sources may suggest the existence of cross-reactivity among allergens from taxonomically related or nonrelated biological sources.

Among the protein components of pollens, those specifically expressed in this tissue have been scarcely analyzed. The availability of new well characterized enzymes or their clones should help the studies of the pollen metabolism, germination, and defense mechanisms. 1,3-β-Glucanases (glucan endo-1,3-β-glu-

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1 The abbreviations used are: IEF, isoelectrofocusing; PR, pathogenesis-related; RP-HPLC, reverse-phase-high performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; ConA, concanavalin A; MALDI-TOF, matrix-assisted laser-desorption ionization time-of-flight; PCR, polymerase chain reaction.

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isocodase, EC 3.2.1.39) are a family of hydrolytic enzymes widely localized in higher plants (27). They have been described as belonging to the pathogenesis-related (PR) 2 group of the PR protein superfamily and exist as multiple forms that differ in size, primary structure, isoelectric point, cellular localization, pathogen induction, and pattern of regulation. Although the expression of many 1,3-β-glucanase genes is induced in response to pathogen attack (28, 29), other genes are constitutively expressed in seeds, roots, and floral tissues of healthy plants (30, 31). Those present in floral tissues, such as Tag1 and Sp41 from tobacco, are developmentally regulated and unrelated to defense response (32, 33). Although 1,3-β-glucanases have been found in sepal, ovary, pedicel, style, and anther (32, 34–38), they have not been detected at significant levels in pollen tissue (32, 33, 36). Finally, active 1,3-β-glucanase has also been found in banana fruit (39), and allergenic isoforms have been described in latex extracts (40).

We herein describe the isolation and characterization of an olive pollen allergen, Ole e 9, which exhibits high clinical significance. Its identification as an active pollen-specific 1,3-β-glucanase as well as its amino acid sequence is also reported.

**EXPERIMENTAL PROCEDURES**

**Isolation of Olive Pollen Allergen**—Olive tree pollen was obtained from Biopol (Spokane, WA) lot No. 97-0524 containing 0.17% foreign pollen and 0.68% other plant parts. The extract was prepared as described previously (9). The lyophilized saline extract was dissolved in 0.2 M ammonium bicarbonate and fractionated on a size exclusion Sephacry G-150 column equilibrated in the same solution. Fractions containing proteins in the range of 30–60 kDa were pooled and rechromatographed on the same column. IgE binding activity of the fractions obtained in all the chromatographic steps were tested by immuno blotting using sera from patients that were allergic to olive tree pollens. Positive samples were applied onto a phenyl-Sepharose column equilibrated in 50 mM Tris-HCl, pH 7.5, containing 0.5 M pollen. IgE-positive samples were applied onto a phenyl-Sepharose blotting using sera from patients that were allergic to olive tree pollens. The proteins transferred to nitrocellulose membranes were immunostained as described previously (7). The membranes were incubated with a pool of sera from patients allergic to olive pollen (diluted 1:10) followed by reaction with mouse anti-human IgE (diluted 1:5000) kindly donated by Dr. M. Lombardero (ALK-Abello®). Afterward, blots were incubated with horseradish peroxidase-labeled goat anti-mouse IgG (Pierce) diluted 1:5000 in phosphate-buffered saline, 0.05% Tween 20, 1% skim milk. The peroxidase reaction was developed using the ECL Western blotting reagent (Amersham Pharmacia Biotech) as described previously (7).

**Immunoblotting and Specific IgE Quantitation**—IgE-reactive proteins transferred to nitrocellulose membranes were immunostained as described previously (7). The membranes were incubated with a pool of sera from patients allergic to olive pollen (diluted 1:10) followed by reaction with mouse anti-human IgE (diluted 1:5000) kindly donated by Dr. M. Lombardero (ALK-Abello®). Afterward, blots were incubated with horseradish peroxidase-labeled goat anti-mouse IgG (Pierce) diluted 1:5000 in phosphate-buffered saline, 0.05% Tween 20, 1% skim milk. The peroxidase reaction was developed using the ECL Western blotting reagent (Amersham Pharmacia Biotech) as described previously (7).

**Molecular Mass Determination**—The molecular mass of the isolated allergen was determined by using the Protean IEF system (Bio-Rad) according to the manufacturer’s protocol. Molecular mass determination was estimated according to the molecular masses of standards used. The protein concentration of the isolated allergen was determined by using the SMART® RACE cDNA amplification kit (CLONTECH) following the user manual.

**PCR-based Cloning of the cDNA for Ole e 9**—A portion of the 1,3-β-glucanase gene from the pathogenesis-related (PR) family was amplified from total RNA prepared from olive tree pollen using the SMART® RACE cDNA amplification kit (CLONTECH) following the user manual.

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OLSF and the SMART II primer. The confirmation of the nucleotide sequence of several segments needed the use of the OLB9 and OLB9E primers. These partial amplifications allowed the design of the primers NTC-OL9, NT-OL9, and CT-OL9 to determine the full-length of the nucleotide-coding sequence of Ole e 9. The DNA nucleotide sequencing was carried out according to Sanger et al. (48) adapted for PCR.

The theoretical prediction of the processing site was achieved according to Nielsen et al. (49). Homology searches were performed using the BLAST network based on Altschul et al. (50). Multiple amino acid sequence alignments were performed using the CLUSTAL W program (51). Sequence-based secondary structure was predicted according to Garnier et al. (52).

Northern Blot—Total RNA from different olive tissues (pollen, stem, leaf, and fruit) and from different pollens (Syringa vulgaris, Ligustrum vulgare, Lolium perenne, Fraxinus excelsior, Cynodon dactylon, Salosa kali, and Betula verrucosa) were isolated for olive pollen (7). 10 μg of each total RNA was fractionated by electrophoresis in 1% formaldehyde-agarose gel and blotted onto Hybond-N nylon membranes (Amersham Pharmacia Biotech) using standard methods (53). Membranes were prehybridized with hybridization buffer (Ambion). A 701-base pair fragment, generated from olive pollen cDNA by (53). Membranes were prehybridized with hybridization buffer (Amersham Pharmacia Biotech) using standard methods (53). Membranes were prehybridized with hybridization buffer (Ambion). A 701-base pair fragment, generated from olive pollen cDNA by (53). Membranes were prehybridized with hybridization buffer (Ambion). A 701-base pair fragment, generated from olive pollen cDNA by (53). Membranes were prehybridized with hybridization buffer (Ambion). A 701-base pair fragment, generated from olive pollen cDNA by (53). Membranes were prehybridized with hybridization buffer (Ambion). A 701-base pair fragment, generated from olive pollen cDNA by (53). Membranes were prehybridized with hybridization buffer (Ambion). A 701-base pair fragment, generated from olive pollen cDNA by (53). Membranes were prehybridized with hybridization buffer (Ambion). A 701-base pair fragment, generated from olive pollen cDNA by (53).

**RESULTS**

Detection of an Olive Pollen Allergen of High Molecular Mass—IgE binding immunoblotting assays with the sera from 72 patients that were allergic to olive revealed the presence of a protein band in the olive pollen extract at 45 kDa that was recognized by 44 sera (61.1%). 16 of these positive sera were selected for the immunological assays performed along the isolation procedure of this new olive allergen. Based on these preliminary prevalence data, this allergen would be in addition to Ole e 1 the most important allergen from olive pollen.

Isolation, Structural Characterization, and Partial Amino Acid Sequencing of Ole e 9—The olive pollen extract was fractionated by size-exclusion chromatography in Sephadex G-150. Frctions containing IgE-binding proteins of molecular mass ranging from 35 to 50 kDa (determined by SDS-PAGE) were pooled and lyophilized. This sample was again chromatographed in the same column to mainly eliminate the dimeric form of Ole e 1 (40 kDa), which interfered in the following steps. The allergenic fractions were further chromatographed on a phenyl-Sepharose affinity column and eluted with distilled water. An analysis of the isolated protein by RP-HPLC rendered two close peaks (Ha and Hb) of the allergenic protein. These samples were independently analyzed for their molecular properties. They exhibited identical molecular mass (46 kDa) in SDS-PAGE (Fig. 2) and values of 46,431 kDa and 46,307 kDa by MALDI-TOF mass spectrometry. A high molecular mass band observed in SDS-PAGE (Fig. 2, lanes Ha, Hb, and a–) was related to the dimer form of the allergen, because it was converted to the monomer form after treatment with 2-mercaptoethanol (Fig. 2, lane a+). The amino acid composition of the two HPLC-separated peaks showed a near identity (data not shown). Edman degradations of these two polypeptides were unsuccessful, but tryptic digestion and RP-HPLC separation of the soluble products rendered indistinguishable peptide maps. Therefore, these two allergenic peaks separated by HPLC would be isomers of the same protein. The protein eluted from the phenyl-Sepharose column was analyzed in IEF, rendering four bands with pI values of 4.8, 4.9, 5.1, and 5.4. According to the standard nomenclature of allergens (54), this protein was called Ole e 9.

The amino acid sequences of seven tryptic peptides of Ole e 9 were determined by Edman degradations. The alignment of these sequences with those of proteins contained in the GenBankTM/EMBL data base revealed a homology with 1,3-β-glucanases.

The purified Ole e 9 binds to the lectin ConA (Fig. 2B), indicating the presence of mannose-containing glycans in the olive allergen. In this regard, the presence of glycan moieties in several 1,3-β-glucanases from plants has been suggested (55).

**Enzymatic Activity of Ole e 9**—The potential 1,3-β-glucanase activity of the allergen was measured by using laminarin as a substrate. Purified Ole e 9 exhibited a specific activity of 38.9 ± 5.6 mg of glucose released/min/μmol of protein at pH 5.0. The optimum pH is maintained from ~pH 4.5 up to at least 6.0 (Fig. 3).

**Immunoochernical Characterization of Ole e 9**—After SDS-PAGE and the transference to membranes of nitrocellulose, the protein was immunostained with a pool of sera from patients allergic to olive pollen. The sample obtained from the phenyl-Sepharose column rendered a single IgE-reactive band of 46 kDa apparent molecular mass, whereas the protein eluted from HPLC column also showed the dimer at 91 kDa, which disappeared after a reduction of disulfide bridges (Fig. 2C).

The IgE binding to purified Ole e 9 was assayed by enzyme-linked immunosorbent assay with 46 sera, which were randomly selected from a population of patients allergic to olive pollen (Fig. 4). The IgE binding to Ole e 1 was also analyzed with the same sera for comparison. The clinical prevalence results were 65 and 75% for Ole e 9 and Ole e 1, respectively, corroborating that Ole e 9 is a major allergen from olive pollen with an incidence near to that of Ole e 1.

**Cloning Strategy and Characterization of the Primary Structure of Ole e 9**—Based on the amino acid sequences of the Ole e 9 tryptic peptides, several oligonucleotides were designed to be used as primers in the cloning strategy. NTC-OL9 and CT-OL9 were those finally selected to determine the full-length of the nucleotide sequence coding for Ole e 9 (Fig. 5). The deduced amino acid sequence corresponded to an open reading frame of 460 residues in length. Because a putative signal peptide is predicted at the N-terminal region of the polypeptide (52), we took advantage of sequences from the homology profile of the protein encoded by the nucleotide (52), selected from the hydropathy profile (data not shown), we applied the rules of Nielsen et al. (49) to detect the cleavage position of signal peptides, obtaining the processing site of this protein between Ser-26 and Gln-27. Considering Gln-27 as the N-terminal end of the mature protein, a new PCR amplification reaction was performed (Table I) to obtain exclusively the sequence of the potential mature protein. Eight complete clones

![Fig. 2. SDS-PAGE analysis along the purification steps of Ole e 9. A. Coomassie Blue staining of olive pollen crude extract (lane E), sample eluted from SephalDEX G-150 (lane S), sample eluted from phenyl-Sepharose column (lane P), and protein fractions eluted from RP-HPLC (lanes Ha and Hb). B, staining of fraction H with ConA lectin after SDS-PAGE and transfer to membranes. C, immunostaining with sera from olive allergic patients of fractions from phenyl-Sepharose column (lane P) and fraction RP-HPLC treated (lane a+) or untreated (lane a−) with 2-mercaptopetanol. M, molecular mass markers. Gels in A and C were performed in 12% polyacrylamide gel electrophoresis, and gel in B was run in 15% polyacrylamide gel electrophoresis.](image-url)
were sequenced, four of them containing the putative signal peptide and the other four without such a sequence.

A low degree of polymorphism has been detected in the amino acid sequence of Ole e 9, 98% identity among clones including the signal peptide and 99–100% identity for clones codifying the putative mature protein. The deduced amino acid sequence includes two potential N-glycosylation sites (NX(S/T)) at positions Aas-355 and Aas-447 in agreement with the positive reaction of Ole e 9 with the ConA lectin (Fig. 2B). The presence of glutamine at the N-terminal position of the mature protein, which spontaneously cycles to pyroglutamate, can explain the unsuccessful reaction of the allergen with the Edman reagent. The molecular mass and pl of Ole e 9, calculated from the amino acid sequence excluding the putative signal peptide, were 46,044 Da and 5.62, respectively, which essentially agreed with those of the purified protein. The deduced amino acid composition also agreed with that obtained by acid hydrolysis of the purified protein (data not shown).

The alignment of the sequence of Ole e 9 with the 1,3-β-glucanase family (Fig. 6) confirmed the proposed homology. Ole e 9 showed 39, 33, 32, and 31% identity to 1,3-β-glucanase from wheat roots (56), to SgGN1 from male catkin of willow (38), and to anther-specific 1,3-β-glucanases (A6 proteins) from Arabidopsis thaliana and rapeseed (36), respectively. Interestingly, all the catalytic residues of 1,3-β-glucanases are conserved in Ole e 9 (Fig. 6). The common feature of these proteins, including Ole e 9, is a long Cys-enriched C-terminal extension (108–112 amino acid residues), which is absent in the remaining 1,3-β-glucanases (35, 55, 57, 58) such as those from barley and sp41 from tobacco style (Fig. 6). In the common N-terminal region (positions 1–352), Ole e 9 exhibits 39 and 34% amino acid sequence identity to these 1,3-β-glucanases from barley and tobacco, respectively.

A predictive analysis of secondary structure of Ole e 9 was performed on the basis of the amino acid sequence (Fig. 5). The calculated percentages of the main elements of secondary structure rendered 19% α-helix, 23% β-strand, and 58% aperiodic conformation. The pattern shows an alternation of α-helix and β-strand motifs at the 350 first amino acid residues of the polypeptide chain, which is a typical conformational structure ("α/β barrel" or Triose phosphate isomerase barrel) of glycosyl-hydrolases (59), supporting once again that Ole e 9 is a member of this family of proteins.

Expression of Ole e 9 in Olive Tissues and Other Pollen Species—The availability of cDNA clones allowed the study of the expression of the olive pollen 1,3-β-glucanase in different tissues. Total RNAs were isolated from leaves, fruit, and stem, and subjected to Northern blot analysis. The probe used was a PCR product (701 base pairs) amplified with OL9C and OL9A, which corresponded to the polypeptide segment comprised between positions Thr-160 and Tyr-393 of the protein. As shown in Fig. 7A, a single hybridizing band corresponding to a mRNA of approximately 1580 base pairs was identified exclusively in pollen tissue.

The same radiolabeled probe mentioned above was used by Northern blot to analyze the existence of proteins homologous to Ole e 9 in different allergenic pollens. Total mRNAs extracted from Oleaceae pollens (ash, lilac, and privet), ryegrass, Bermuda grass, mugwort, S. kali, and birch were analyzed (Fig. 7B). No mRNA expression was detected in pollens taxonomically unrelated to olive. The intensity of the signal varied for pollens of the Oleaceae family. Ash pollen expressed a detectable transcript product, whereas privet and lilac pollen did not give a hybridizing band. These results suggest the existence of a low degree of conservation in the primary structures of potential counterparts of Ole e 9 present in other pollens.

**DISCUSSION**

Most of the allergenic pollens contain a number of proteins usually with molecular masses of <50 kDa that bind to IgE from the sera of patients sensitive to such a source of pollen. Olive pollen displays one of the most complex allergenic patterns known so far. In fact, >20 allergenic bands can be detected in olive pollen extracts, and eight of them have been isolated and characterized. They exhibit a degree of prevalence ranging from ~5% for Ole e 8 to >70% for Ole e 1 and molecular masses comprised between 5.8 kDa for Ole e 6 and 32 kDa for Ole e 4. A new glycosylated allergen, Ole e 9 (46.4 kDa), now has been isolated and characterized as a 1,3-β-glucanase.

1,3-β-Glucanases are widely distributed in higher plants, but their biological function is still unclear. Although many 1,3-β-glucanases have been described as PR proteins because of their induction by pathogens, others have been found to be constitutive enzymes (PR-like proteins) expressed during physiological and developmental processes of healthy plants, such as germination, senescence, fertilization, and responses to environment.
tal conditions (55, 58, 60, 61). Based on the length of the polypeptide chain, two types of 1,3-β-glucanases have been described: 1) short 1,3-β-glucanases, which exhibit basic or acidic pI, and molecular masses comprised between 33 and 41 kDa (300–350 amino acids) and 2) long 1,3-β-glucanases with molecular masses of 45 kDa (420–480 amino acid residues) and being moderately acidic (1,3-β-glucanase from wheat A. glutineum intoxicated roots (56)) or basic (A6 proteins from A. thaliana and rapeseed anthers (36) and SgGN1 from willow male catkins (38)). Ole e 9 was acidic and long but exhibited an amino acid sequence identity to the wheat enzyme limited to 39%. These data, as well as the pollen-specific location and the constitutive expression of the protein, suggest that Ole e 9 can be a novel class of 1,3-β-glucanases in plants.

Short basic 1,3-β-glucanases exhibit a large variability of substrate specificity with a narrow optimum pH at approximately 4.5 and enzymatic activities that appear to be 50–250 times higher in degrading the 1,3-β-glucan substrate laminarin than most of the acidic enzymes (55, 62). However, no catalytic activity so far has been associated with long 1,3-β-glucanases. In fact, no enzymatic activity was detected for A6 tapetum-specific 1,3-β-glucanases (36), which was explained because of the change of Glu-331 (refer to Fig. 6) to Gln in the polypeptide chain (62). In contrast, Ole e 9 possesses the tetrad of ionizable amino acids (Glu-268, Glu-322, Lys-325, and Glu-331 positions corresponding to Olee9i NcFig. 6) that have been proposed to be involved in the catalytic mechanism of these glycan endohydrolases (62). This conservativeness may be responsible for the 1,3-β-glucanase reactivity of Ole e 9.

Ole e 9 exhibited a low but significant polymorphism, which was detected by RP-HPLC and confirmed by nucleotide sequence. This property has been frequently described in allergens as well as in 1,3-β-glucanases (both for acidic and basic forms and for vacuole and secreted enzymes). The polymorphism of Ole e 9 may be the result of microheterogeneity not only in the polypeptide chain but in the glycan moiety as well.

![Fig. 5. Nucleotide sequence of cDNA encoding Ole e 9 and deduced amino acid sequence.](http://www.jbc.org/)
occurs for Ole e 1 (7, 63). Ole e 9 possesses two putative N-glycosylation sites both located at the C-terminal extension, and at least one of them is occupied. Both single or double occupation of the potential glycosylation sites as a heterogeneous glycosylation of each site can contribute to the polymorphism of the protein.

The deduced amino acid sequence of Ole e 9 contains a putative signal peptide, suggesting that this 1,3-β-glucanase may be secreted. Among a recent classification of 1,3-β-glucanases (55), those of Class I are PR-2e proteins and accumulate in vacuoles; they are produced as pre-proproteins with a N-terminal hydrophobic signal peptide and a C-terminal glycosylated extension, both being removed during the maturation of the protein (55, 57). On the other hand, extracellular 1,3-β-glucanases (Classes II–IV) are acidic proteins without the C-terminal extension and have been related to developmental

| Oligomer | Nucleotide sequence | Strand | Amino acid sequence |
|----------|---------------------|--------|--------------------|
| OL9A     | 5'-cgaattctRTARTDRTNCCNGT-3' | Antisense | TGNINY (385–390) |
| OL9B     | 5'-cgcctcagACNGNNAAYTHAAYTAY-3' | Sense | TGNINY (385–390) |
| OL9C     | 5'-ACNGTNCAYCAATGGCNGT-3' | Sense | TVHMAV (160–167) |
| OL9D     | 5'-ACGGGAGGACTCCCAATGAGCTT-3' | Sense | HGGDSNEY (273–280) |
| OL9E     | 5'-AAGGTGCATATTGGATCTCCTGGA-3' | Antisense | PQSANDAP (192–199) |
| OL9F     | 5'-TGCCAAATGTCAGGCTTGGCT-3' | Sense | QPTPDOLA (213–220) |
| OL9G     | 5'-TGATAGATGTCAGGACATGAGCTG-3' | Sense | VIGFGOND (80–87) |
| OL9H     | 5'-GCTGACAAATGAGGACATGAGCTG-3' | Sense | GDTXKALL (183–190) |
| NT-OL9   | 5'-GCTGACAAATGAGGACATGAGCTG-3' | Sense | MAATXOQ (1–7) |
| NT-OL9   | 5'-GCTGACAAATGAGGACATGAGCTG-3' | Sense | QPSFLG (27–31) |
| CT-OL9   | 5'-GCTGACAAATGAGGACATGAGCTG-3' | Antisense | PSSGN (456–460) |

**Table I**

Oligonucleotides used as primers in PCR and sequencing

In the nucleotide sequence, R represents A/G, D represents G/A/T, N represents A/C/G/T, Y represents C/T, H represents A/T/C. The noncoding region of the nucleotides are shown in lower case letter. Numbers in parenthesis correspond to the positions of the peptides in the primary structure of the allergen. OL9G and OL9H were used only for sequencing and NT-OL9, NT-OL9, and CT-OL9 were used for cloning the protein with and without the signal peptide. XhoI and NotI sites used for cloning are underlined. SMART II and 3'9-CDS are nonspecific oligonucleotides.

**Fig. 6.** Comparison of amino acid sequences of 1,3-β-glucanases from plant sources. Ole e 9 from olive pollen (Ole9), wheat (Wh-Ta), *Salix gilgiana* (Sg-GN1), *A. thaliana* (At-A6), p41 from tobacco (To-Sp41), and *Hordeum vulgare* (Hor-Ge) (GenBank™ accession numbers AF249675, P52409, AB029462, Q06915, P23432, and Q02438, respectively). Gaps (−) were introduced for optimizing the alignment. Residues conserved in all of the proteins are shaded in black. Residues conserved in four of the chains are shaded in gray. The putative processing site is indicated by an arrow. Potential N-glycosylation residues are shown with an arrowhead, and residues involved in the catalytic activity are identified by asterisks.
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and physiological processes of healthy plants (30–35, 55). Ole e 9 contains a putative signal peptide that should be cotranslationally removed but conserves the C-terminal extension (also glycosylated) in the mature protein. Interestingly, wheat roots and A6 tapetum-specific 1,3-β-glucanases seem likely to conserve the C-terminal extension in the mature protein, because an A6 protein-specific polyclonal antibody detected proteins of 56 and 60 kDa, respectively, in Al-intoxicated wheat roots (56) and rapeseed buds (36). Although the molecular mass of purified Ole e 9 is significantly lower (46.4 kDa), these data agree with our results, because a sequenced tryptic peptide can be aligned at the C-terminal region of Ole e 9. In this sense, it cannot be discarded that Ole e 9 could suffer a cotranslationally processing of a short peptide at the C-terminal end of the molecule, because the molecular mass obtained for the natural protein (46,431 and 46,307 Da) does not exactly fit with that deduced from the nucleotide sequence (46,044 Da) plus the molecular mass of the smallest oligosaccharide structure (NacGlcMan core of 892 Da) attached to a single potential N-glycosylation site.

Several PR-like 1,3-β-glucanases are specifically expressed in reproductive tissues, Tag1 and Sp41 in andthers and style from tobacco, respectively (32, 33, 35, 57), A6 proteins in tapetum from rapeseed and A. thaliana (36), and SgGlN1 in male catkin supposedly in andthers from willow (38). It has been proposed that the expression of the genes from A6 proteins and SgGlN1 is related to the developmental stage of pollen grains (36, 38), and the former may be part of the callase enzyme complex (36). However, no 1,3-β-glucanases have been found so far in pollen. Ole e 9 seems likely to be specifically expressed in this tissue and does not appear to be induced by pathogen infection. As other olive pollen allergens, Ole e 9 is highly soluble and easily released from the pollen after hydration. Whether this 1,3-β-glucanase plays or does not play a role in pollen germination and tube growth remains to be analyzed, but a defense activity cannot be discarded.

Ole e 9 is the first acidic and long 1,3-β-glucanase described with allergenic properties, but a short and basic 1,3-β-glucanase from latex has been demonstrated to induce allergy. In fact, 1,3-β-glucanases have been found in the latex of the rubber tree Hevea brasiliensis (40, 64). They exhibited high capability to bind to IgE antibodies from the sera of patients that were allergic to latex and constitute the latex allergen Hev b 2 (65, 66), which exhibited isoforms of 35–38 kDa and a basic pi (>10). Although food and latex allergens have different ways of access to hypersensitive patients by ingestion and direct contact, respectively, latex 1,3-β-glucanases were found to be strongly recognized by IgE from sera of atopic subjects that were allergic to tomato, potato, and banana (66). The association between latex allergy and hypersensitivity to fruits has been termed “latex-fruit syndrome” (67), and 1,3-β-glucanases could be involved. However, 1,3-β-glucanases have not been explored so far as allergens in vegetable foods despite the fact that these enzymes have been shown to be expressed in such vegetable species. Recently, an abundant and active 1,3-β-glucanase has been isolated from the pulp of ripe bananas (39). The ubiquity of 1,3-β-glucanases in higher plants suggests that they could be a novel type of panallergens able to induce cross-reactivity among vegetable sources (66). Now, this statement can be extended to aeroallergenic pollens, thus contributing to explain the existence of cross-reactivity between pollens and fruits. Because 1,3-β-glucanases constitute a broad family of proteins, the degree of similarity in their amino acid sequences will lead to different cross-reactivities.

The finding of Ole e 9 as a relevant allergen in olive pollen again supports the involvement of PR and PR-like proteins in IgE-mediated allergy from which there are significant examples (68). Finally, because 1,3-β-glucanase-encoding genes are frequently included for expression of defense-related proteins when producing genetically engineered plant foods of economic interest, their putative allergenicity should be taken into account.

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