INTRODUCTION

Olive (Olea europaea L.) pollen is one of the main causes of allergy by inhalation in Mediterranean countries and some areas of North America (Bousquet et al., 1985; Wheeler, 1992). The major allergen of this pollen (Ole e I) has been isolated, purified and biochemically characterized (Villalba et al., 1990, 1993). Cloning and sequencing of several cDNAs coding for the olive allergen have also been recently carried out (Lombardero et al., 1994; Villalba et al., 1994) (GenBank™/EMBL Data Bank accession numbers S75766 and X76395, respectively). The protein has an acidic nature, with several glycosylation variants (Batanero et al., 1994) as well as microheterogeneity at several positions (Villalba et al., 1993, 1994).

Transmission electron microscopy immunocytochemical localization has been used to trace this protein throughout pollen development (Martín-Orozco et al., 1994; Rodríguez-García et al., 1995a,b). Within the olive pollen grain, the allergenic protein is specifically located in the cisternae of the rough endoplasmic reticulum (RER). It has been reported that this RER system undergoes conspicuous ultrastructural changes during olive pollen grain development. RER cisternae progressively transform into abundant pockets or sacs with an electron-dense content, scattered throughout the cytoplasm in the mature pollen stage (Rodríguez-García and Fernández, 1990).

Common epitope determinants to Ole e I have also been found in several species of the Oleaceae family, sharing similar localization (Martín-Orozco et al., 1994; Fernández et al., 1996). The amino acid sequence of Ole e I shows a remarkable similarity to the polypeptides encoded by the LAT52 gene from tomato pollen (36% identity) and by the Zmc13 gene from maize pollen (38% identity). Both gene products seem to play essential roles in late events of pollen development, such as pollen maturation, hydration, germination and/or pollen tube growth (Hanson et al., 1989; Muschietti et al., 1994). However,
as is also the case for many other allergens, the biological role of these proteins remains unknown. In order to obtain clues regarding the biological function of the Ole e 1, we analyzed the temporal and spatial expression patterns of Ole e 1 gene at different stages of anther development, and demonstrated the involvement of sporophytic tissues of the anther (tapetum) in the expression of this allergen.

MATERIALS AND METHODS

Plant materials
Floral buds from selected Olea europaea L. trees (Var. Picual) in Granada (Spain) were collected during the months of April and May. After dissection and staging of flowers by squashing and light microscopic (LM) observation, the anthers were immediately frozen and stored in liquid nitrogen or fixed and processed for microscopy. However, whole flower buds were used from the pre-meiotic up to the early meiotic prophase stages, as dissection was impracticable due to the small size of the flowers.

Dehiscent pollen grains were collected in large bags by vigorously shaking the inflorescences and were then sieved to separate the grains from debris. Leaf samples were obtained from young plantlets of the same variety grown under controlled conditions in Almería (Spain) and processed as above.

Preparation of protein extracts and immunoblotting
Specimens stored in liquid nitrogen were ground in a mortar with sand and processed as above.

Leaf samples were obtained from young plantlets of the same variety grown under controlled conditions in Almería (Spain) and processed as above.

Preparation of digoxigenin-labeled probe
Ole e 1 cDNA (clone 3c, 438 bp) (Villalba et al., 1994), inserted in pGEM-4Z (Promega), was PCR-amplified using T7 and SP6 primers and digoxigenin-11-dUTP (Boehringer Mannheim) in the reaction mix according to Leitch et al. (1994). The amplified fragment was ethanol-precipitated according to standard protocols (Sambrook et al., 1989), checked by agarose gel electrophoresis and finally quantified.

Light microscopy in situ hybridization
Samples were fixed overnight in ice-cold 0.1 M cacodylate buffer, pH 7.2, containing 4% paraformaldehyde and 0.2% glutaraldehyde. They were then washed in the same buffer, dehydrated in a graded ethanol series and xylene, and embedded in paraffin. Sections (7 μm thick) were attached to 3-aminopropyl triethoxy-silane-coated slides, deparaffinized with xylene and rehydrated through a graded ethanol series. Slides with anther sections were pretreated with 1 μg/ml proteinase K in 100 mM Tris-HCl, 50 mM EDTA, pH 7.5, for 30 minutes at 37°C, followed by 100 mM triethanolamine, pH 8.0 for 10 minutes and 0.25% acetic anhydride in 100 mM triethanolamine, pH 8.0 for 10 minutes, both at room temperature. The sections were then dehydrated in a graded ethanol series and vacuum-dried. For hybridization, each slide was covered with 60 μl of the hybridization solution, containing 2 μl of heat denatured PCR-generated digoxigenin (dig)-labelled probe (ca. 50 ng) in 50% formamide, 300 mM NaCl, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.02% Ficoll, 0.02% PVP, 0.02% BSA, 10% dextran sulphate, 60 mM DTT. Sections were covered with coverslips and incubated in a humid chamber for 20 hours at 42°C. After hybridization, the slides were washed four times in 4× SSC at 42°C for 10 minutes with gentle agitation, and twice in 2× SSC at room temperature for 10 minutes. Detection was carried out after blocking with 3% blocking reagent (Boehringer Mannheim) in phosphate-buffered saline (PBS)-0.1% Tween 20 for 90 minutes by incubating the slides with 1:1000 anti-dig, alkaline phosphatase-conjugated antibody (Boehringer Mannheim) in blocking solution for 1 hour. The slides were then washed twice in detection buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 5 mM MgCl2, 1 mM levamisole) for 5 minutes and the color reaction (using NBT/BCIP substrate) was left to develop for 16 hours. Observations and photographs were made on permanent preparations mounted in DePx. Negative control reactions were set by omitting the probe into the hybridization mix and/or the anti-dig antibody.

Transmission electron microscopy in situ hybridization
Dehiscent pollen and excised anthers at different stages of microsporogenesis were cryofixed without pretreatment by immersion in liquid propane at −180°C in a KF80 unit (Reichert). Freeze
substitution was performed in anhydrous acetone using a Balzers FSU 010 unit (Balzers). The samples were kept at −90°C, −60°C and −30°C for 8 hours at each step and finally brought to −20°C. The samples were infiltrated for 3 months with a graded resin series (Unicryl, BioCell International), and polymerized under ultraviolet light for 3 days at −20°C. Sections (70 nm thick) were cut on a Reichert Jung Ultracut microtome and mounted on formvar-coated nickel grids.

In situ hybridization was performed by floating the grids on 50 µl drops of the corresponding solutions. Proteinase K treatment and hybridization were done as described for LM. Detection of the hybridized probe was carried out after blocking with 5% BSA in PBS by incubation with a sheep anti-digoxigenin antibody conjugated to 10 nm gold particles (BioCell International), diluted 1:100 in PBS. The grids were washed in PBS, and then in water, and stained with 5% uranyl acetate for 10 minutes. Observations were made in a Zeiss EM10C Transmission Electron Microscope operating at 60 kV. Parallel sets of reactions were carried out using chemically fixed material showing comparable results. Negative control sections were treated in the same fashion, but omitting the probe in the hybridization mix.

Immunolocalization

Ultradein sections were prepared as described for transmission electron microscopy (TEM) in situ hybridization. Blocking of non-specific binding sites was carried out by incubation of the sections in 2% BSA, 1% normal goat serum in PBS for 15 minutes. This was followed by treatment with anti-Ole e I monoclonal antibody diluted 1:10 in blocking reagent for 1 hour. The grids were washed in PBS and treated with goat anti-mouse IgG coupled to 20 nm gold particles (BioCell International) diluted 1:50 in PBS for 1 hour. After washing with PBS and ultrapure water, the sections were stained for 10 minutes in 5% uranyl acetate and observed. Negative sections were treated as described, but only blocking reagent instead of the anti-Ole e I antibody solution was used to incubate the grids.

Semithin sections (1 µm thick) from the same blocks prepared for TEM were attached to 3-aminopropyl triethoxy-silane-coated slides and treated as described for TEM immunocytochemistry. After treatment with the secondary antibody, the gold particles were visualized using a silver enhancement kit (BioCell International) according to the manufacturer's instructions, and observed in a Zeiss Axioplan photomicroscope using dark field optics. Negative controls were treated as above but omitting the primary antibody.

RESULTS

Expression of Ole e I in olive anthers at different developmental stages

Fig. 1 shows the results of immunodetection of polypeptides recognized by an anti-Ole e I monoclonal antibody, after blotting the electrophoresed samples from whole bud/anther extracts. The antibody recognized protein bands of 17-19 kDa present in the stages of early microspores, early bicellular pollen and dehiscent pollen. No protein could be detected in early stages of anther development. This result indicates that the protein bands share common epitopes although they may possess different carbohydrate contents. Bands b were detected in the three stages mentioned, while bands a and c were mainly localized at the dehiscent pollen stage (very faint bands a were also occasionally present in the early bicellular pollen stage, not shown). Both the number and the intensity of bands quantitatively increased during pollen maturation stage.

Levels of Ole e I transcripts were analyzed by RT-PCR (Fig.

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\caption{Expression of Ole e I allergen during anther development. Immunoblot of crude protein extracts obtained from anthers at the pre-meiotic stage (lane 1), meiotic prophase (lane 2), metaphase-anaphase I (lane 3), second meiotic division-early tetrads (lane 4), late tetrads (lane 5), early microspores (lane 6), early bicellular pollen (lane 7) and mature (dehiscent) pollen (lane 8). Each sample contained 15 µg of total protein. The blot was developed with monoclonal anti-Ole e I antibody. a and b, glycosylated variants of Ole e I; c, non-glycosylated variant. The positions of marker proteins (kDa) are shown on the left.}
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2A) using total RNA extracted from flower buds/anthers at different stages of development and different plant organs (controls). Equal loading of the samples was ensured by the comparable intensity of bands resulting from RT-PCR amplification of ubiquitin monomers to polyubiquitin pentamers in equivalent samples (Fig. 2B), as ubiquitin mRNAs display steady-state levels in a number of plant species (Watts and Moore, 1989). No signal of Ole e I transcripts was detected using RNA from early stages, nor from control organs such as petals or leaves. A weak band of 612 bp appeared at the late tetrad stage, and increasingly intense bands of the same size were observed at the stages of early microspores and early bicellular and dehiscent pollen. Basic local alignment (Altschul et al., 1990) of the nucleotide sequence obtained for the RT-PCR generated fragment to the nr (all Non-Redundant GenBank+EMBL+DDBJ+PDB sequences, but no EST, STS, GSS or HTGS sequences) database matched those sequences reported for Ole e I by Lombardo et al. (1994) (GenBank accession number S75766) and Villalba et al. (1994) (EMB accession number Y12426) with an identity of 94% over 423 nucleotides (plus 81% over 143 additional nucleotides), and 94% over 392 nucleotides, respectively.

LM in situ hybridization

In order to study the distribution of Ole e I mRNA in specific olive anther tissues and other control cell types, non-radioactive in situ hybridization with a PCR-generated dig-labeled probe was carried out using paraffin sections. No hybridization signal above the background was detected in the pre-meiotic flower bud, nor at early stages of microsporogenesis while meiosis is taking place (Fig. 3A,B). At the late tetrad stage, a signal was located almost exclusively in the tapetal cells. The sporogenous tissue showed only a reduced level of labeling (Fig. 3C,D). High levels of expression were maintained in the tapetum during microspore development (Fig. 3E), although this expression decreased concomitantly with the degeneration of this tissue, which was observed from the early bicellular pollen stage (Fig. 3G,H) to

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\caption{Expression of Ole e I allergen during anther development. Immunoblot of crude protein extracts obtained from anthers at the pre-meiotic stage (lane 1), meiotic prophase (lane 2), metaphase-anaphase I (lane 3), second meiotic division-early tetrads (lane 4), late tetrads (lane 5), early microspores (lane 6), early bicellular pollen (lane 7) and mature (dehiscent) pollen (lane 8). Each sample contained 15 µg of total protein. The blot was developed with monoclonal anti-Ole e I antibody. a and b, glycosylated variants of Ole e I; c, non-glycosylated variant. The positions of marker proteins (kDa) are shown on the left.}
\end{figure}
dehiscence. A progressive accumulation of Ole e I transcripts was observed within the microspores throughout their development (Fig. 3E-H). Higher magnification of sectioned microspores allowed the detection of the signal to be localized in the microspore cytoplasm, while the nucleus, nucleolus and the microspore wall were devoid of labeling (Fig. 3F), thus demonstrating the specificity of the signal.

No color reaction was found in parallel sections where the Ole e I probe (Fig. 3I) or the anti-dig antibody (not shown) were omitted.

TEM in situ hybridization
In situ hybridization of an Ole e I probe to ultrathin sections of developing olive microspores showed gold particles decorating both the cytoplasmic and the RER-associated ribosomes at the early microspore stage (Fig. 4A). Intense labeling was observed in the RER stacks or pockets within the cytoplasm of both the vegetative and the generative cells in the bicellular and mature pollen stages (Fig. 4B). No labeling was detected in the pollen wall, vegetative and generative nuclei, nor in organelles, vacuoles or lipid bodies in any of the stages tested. No significant gold labeling was found in negative control sections when the same treatment was applied, but the probe was omitted (not shown).

Ole e I immunolocalization
In order to determine the precise localization of the major olive allergen, both LM and TEM immunocytochemical studies were carried out in the same developmental stages that were used for expression studies. The localization observed coincided with results previously published by our group (Martín-Orozco et al., 1994; Rodríguez-García et al., 1995a,b). However, the use of low-temperature processing methods for the preparation of samples allowed the ultrastructural localization of Ole e I in the pollen wall of mature olive pollen (Fig. 5A). Noticeable gold labeling was detected in the outer part of the pollen exine (ectexine) whereas the inner part (endexine), the intine and the aperture region appeared devoid of gold particles. Negative controls prepared by omitting the primary antibody showed no significant labeling (Fig. 5B). Furthermore, additional labeling appeared in the tapetal tissue during the microspore stage after immunocytochemical detection with LM using the antibody and silver enhancement, thus showing the protein to be present in this tissue (Fig. 5C,D).

DISCUSSION
Three Ole e I isoallergens are identified at late stages of pollen development
Previous studies carried out in our group (Martín-Orozco et al., 1994; Rodríguez-García et al., 1995a,b; Fernández et al., 1996) have described the immunocytochemical localization of this allergen in the cisternae of the rough endoplasmic reticulum during the late stages of pollen development. The immunoblot experiments carried out here confirm the presence of the allergen in the same stages and also determine quantitatively the relative abundance of the three forms of this protein reacting with the antibody. The presence of microheterogeneities in both the amino acid and the corresponding nucleotide sequence of this protein have also been widely reported (Villalba et al., 1993, 1994; Lombardero et al., 1994), explaining the high degree of polymorphism exhibited by the natural protein. This high degree of polymorphism is a characteristic of plant pollen...
allergens such as grass (Johnson and Marsh, 1965), short ragweed (Bond et al., 1991; Griffith et al., 1991) and birch (Swoboda et al., 1995) among others. Furthermore, in the case of olive, the presence of isoallergens is also attributed to glycosylation variants (Lauzurica et al., 1988a,b; Villalba et al., 1990; Batanero et al., 1994). These observations enhance the importance of further characterization studies in order to obtain well defined and homogeneous olive allergen molecules for use in multiple applications, such as diagnosis, therapy and research.

**Ole e I gene expression is probably regulated at the transcriptional level**

Former northern blot analyses conducted on RNA from pollen, leaf, stem and fruit tissues of olive tree showed *Ole e I* transcripts to be present only in mature pollen (Villalba et al., 1994) and not in the other tissues tested. However, no data concerning the temporal and spatial pattern of expression of this gene during pollen development were available to date. The immunoblotting experiments described in this paper show that the allergen is present in whole anthers from the early
Fig. 4. TEM in situ hybridization of *Ole e 1* probe to olive anthers containing microspores at different developmental stages. (A) Early microspore stage. (B) Mature pollen. The hybridization signal is found in the cytoplasm, frequently associated to ribosomes of the endoplasmic reticulum. Cytoplasmic organelles, microspore and pollen wall and nuclei are devoid of labeling. ER, endoplasmic reticulum; EX, exine; L, lipid body; M, mitochondria; N, nucleus; P, plastid; arrows, gold labeling. Bars, 0.5 μm.
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Ole e I allergen during olive anther development

However, these experiments do not discriminate whether or not the protein was present in the developing microspores or in the tapetum. Because ultrastructural studies carried out in our group failed to localize the protein within early microspores, it can be assumed that early appearance of the protein takes place in the tapetum at this stage, as is also confirmed here by LM immunolocalization. Both RT-PCR analysis and in situ hybridization experiments determined that Ole e I transcripts are already present in the anther at the late tetrad stage, and display a pattern similar to that of the allergen: mRNAs accumulate primarily in the tapetum, and then become more abundant within the microspores, concomitantly with the degeneration of the tapetum and the maturation of microspores. Both the transcripts and the protein accumulated massively in dehiscent pollen. These results suggest that the expression of this gene is likely to be regulated at the transcriptional level. However, there is a delay in the appearance of the protein at the stages of late tetrad/early microspore that could be explained by the particular cycle of rRNA taking place during pollen development. The population of cytoplasmic ribosomes is strongly depleted during meiotic prophase (Mackenzie et al., 1967), while a repopulation takes place at the end of meiosis, coincidentally with the initiation of the haploid phase of expression. It has been postulated that structures called nucleoloids could be involved in the buildup of the post-meiotic ribosome population (Dickinson and Heslop-Harrison, 1970). Ultrastructural studies in olive microspores show the number of ribosomes still clearly diminished during the early microspore stage, where nucleoloids are frequently observed (Alché et al., 1994). In our opinion, the absence or reduction in the machinery for translation at the stages of late tetrad and early microspore could better explain the absence of the protein than the presence of a post-transcriptional regulatory mechanism.

Synthesis and storage of Ole e I takes place in the RER within the pollen grain

Allergenic proteins have been localized in the pollen of a number of allergenic species, such as ryegrass (Smart and Knox, 1980; Staff et al., 1990), birch (Grote, 1991) and timothy grass (Grote et al., 1994). However, no studies published to date have reported the RER as the site of antigenic-protein localization. Immunocytochemical localization of Ole e I (Rodríguez-García et al., 1995a,b) convincingly documented that the RER system is involved in the storage of allergenic proteins in several Oleaceae species. It was also proposed that the RER could be the synthesis site for these proteins, following the widespread pattern of synthesis by ribosomes and co-translation into the lumen of the RER (Larkins and Hurkman, 1978; Oparka and Harris, 1982; Krishnan et al., 1986). This appears to be confirmed by the result of the TEM in situ hybridization experiments shown in the present study.

The use of cryotechniques allows the identification of Ole e I in both the tapetum and pollen ectexine

Contrary to Bet v I, the major birch pollen allergen transcripts which were absent from sporophytic anther tissues of any developmental stage (Swoboda et al., 1995), the presence of Ole e I transcripts has been clearly demonstrated in the tapetum of olive anthers, from the late tetrad stage until the degeneration of this tissue during pollen maturation. The
tapetum has been largely considered as a putative source from which pollen wall proteins and proteins responsible for allergy originate (Knox et al., 1970; Knox and Heslop-Harrison, 1971; Pacini, 1994). Allergic proteins have also been widely localized in the pollen wall (Staff et al., 1990; Grote, 1991; Grote at al., 1994). However, pioneer immunocytochemical localization of Ole e I failed to demonstrate the presence of the allergen in the olive pollen wall, whereas the tapetum remained unexplored because of the technical difficulties in the appropriate processing of this tissue for immunolocalization. The use of cryotechniques as reported here provided a number of advantages, including a better preservation of the antigenicity of the samples. It also prevented washing out of the soluble protein from the outer pollen wall (ectexine) by diffusion to the aqueous chemical fixative used in our previous papers. We demonstrate here the presence of the major olive allergen in both the pollen wall and the tapetum, therefore confirming the involvement of this sporophytic tissue in the synthesis and accumulation of this allergenic protein in the pollen grain. Subcellular localization of the protein in the tapetum could not be performed as the preservation of the structure was still very poor. Ultrastructural preservation techniques have yet to be optimized for this tissue.

**New insights into the biological function of Ole e I**

Putative functions of Ole e I have been deduced from the antisense repression experiments carried out by Muschietti et al. (1994) for LAT52 gene product, for which Ole e I sequence shares a relatively high homology. These authors described that reduced expression of LAT52 gene correlated with abnormal pollen function, and suggested that this protein plays a role in pollen hydration and/or pollen germination. Pollen hydration occurs because of differences in water potential between the stigma (or the medium, in an in vitro assay) and the pollen (Heslop-Harrison and Shivanna, 1977). The high content of Ole e I protein within the pollen grain (up to 20% of the total amount of protein) suggests a potential role for Ole e I in determining the osmotic gradient needed for hydration. A detailed analysis of Ole e I protein and its transcripts during in vitro hydration and pollen tube growth is currently being carried out in our laboratory.

We are grateful to Dr C. Lahoz (Departamento de Immunología, Fundación Jiménez Díaz, Madrid, Spain), for kindly providing the monoclonal antibody to Ole e I, and to Ms C. Martínez-Sierra for technical assistance. This work was supported by the European Commission, Contract FMBI-CT95-0470 and by DGES (Spanish MEC) Project PB95-0080.

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