

# Ole e 1, the Major Allergen from Olive (*Olea europaea* L.) Pollen, Increases its Expression and is Released to the Culture Medium during in vitro Germination

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**Ole e 1 is a well-characterized allergenic protein from olive pollen. This paper examines its presence and that of its transcripts during in vitro pollen germination and pollen tube growth. A significant increase of the protein was detected after the emergence of the pollen tube, whereas part of the protein was released into the culture medium throughout pollen germination. A slight increase in the number of Ole e 1 transcripts was also detected prior to the described rise in the protein level. Within the pollen tube, the allergen was localized in the subapical region, mainly in the lumen of endoplasmic reticulum cisternae. Ole e 1 was also localized extracellularly in the vicinity of the pollen tube cell wall. These findings are discussed regarding the biological role attributed to the protein during pollen hydration and pollen tube growth and in terms of their importance for the understanding of the allergenic response in humans. On the basis of recent findings for the LAT52 protein in tomato, we propose that the homologous Ole e 1 protein might participate in a similar signal transduction pathway in olive, to control pregermination and pollen tube emergence and guidance.**

**Keywords:** Allergen — In vitro germination — Ole e 1 — *Olea europaea* — Olive tree — Pollen.

## Introduction

The major functions of the male gametophyte in flowering plants include the production of two sperm cells and their transport within the pollen tube through style and ovary tissues into the embryo sac in the ovule, where they participate in double fertilization (Mascarenhas 1993). The molecular events occurring during these processes are important topics of study, since they are involved in variations in the pollen wall structure, the cytoskeleton, interactions between the pollen tube and the female flower structures, incompatibility and gamete competition.

Efforts have been made to isolate and characterize those genes expressed during pollen development and pollen germination, particularly focusing on the assignment of functions to

these gene products. Pollen allergens can be considered gene products of presumed relevance to pollen physiology in addition to their allergenic nature for humans. The study of these allergens has also deepened our understanding of basic aspects of pollen biology by providing useful tools such as antibodies, sera, sequences and probes.

Olive (*Olea europaea* L.) pollen allergy has been reported to affect more than 20% of the population in those Mediterranean countries in which the crop is widely cultivated (Bousquet et al. 1985, Gioulekas et al. 1991, Wheeler 1992, Florido et al. 1999). To date, ten allergens (referred to as Ole e 1 to Ole e 10) have been isolated from olive pollen and characterized. Ole e 1 is considered to be the major allergen, affecting more than 70% of patients hypersensitive to olive pollen (Rodríguez et al. 2002). It presents a number of glycosylation forms which include a 18.4-kDa non-glycosylated form and a 20-kDa glycosylated form. Another 22-kDa hyperglycosylated variant, as well as a 40-kDa variant composed of dimers of the glycosylated forms, are sometimes present in the protein preparations (Villalba et al. 1993, Villalba et al. 1994). The amino acid sequence of Ole e 1 displays relevant (24–34%) homology to pollen proteins from maize, tomato, ryegrass, birch, rice, *Arabidopsis* etc. Identity rises to >85% when it is compared to the sequences of Ole e 1-like proteins from members of the *Oleaceae* family (lilac, privet, ash, forsythia) (Obispo et al. 1993, Martín-Orozco et al. 1994, Batanero et al. 1994a, Batanero et al. 1996). Ole e 1 itself exhibits microheterogeneities at several positions of its amino acid sequence (Villalba et al. 1993, Villalba et al. 1994, Lombardero et al. 1994).

Research is now beginning to reveal the biological function of Ole e 1. The distribution of the protein and of its transcripts has been studied in anther tissues throughout their development (Alché et al. 1999). This investigation indicates that (i) both synthesis and storage of Ole e 1 take place in the pollen grain endoplasmic reticulum; (ii) the allergen is also located in the pollen ectexine at the mature stage and (iii) the tapetum is involved in Ole e 1 synthesis.

Clues into the putative function of Ole e 1 may be emerging from the antisense repression experiments performed on the Ole e 1-homologous *LAT52* gene product from tomato (Muschiatti et al. 1994) and from recent findings regarding the

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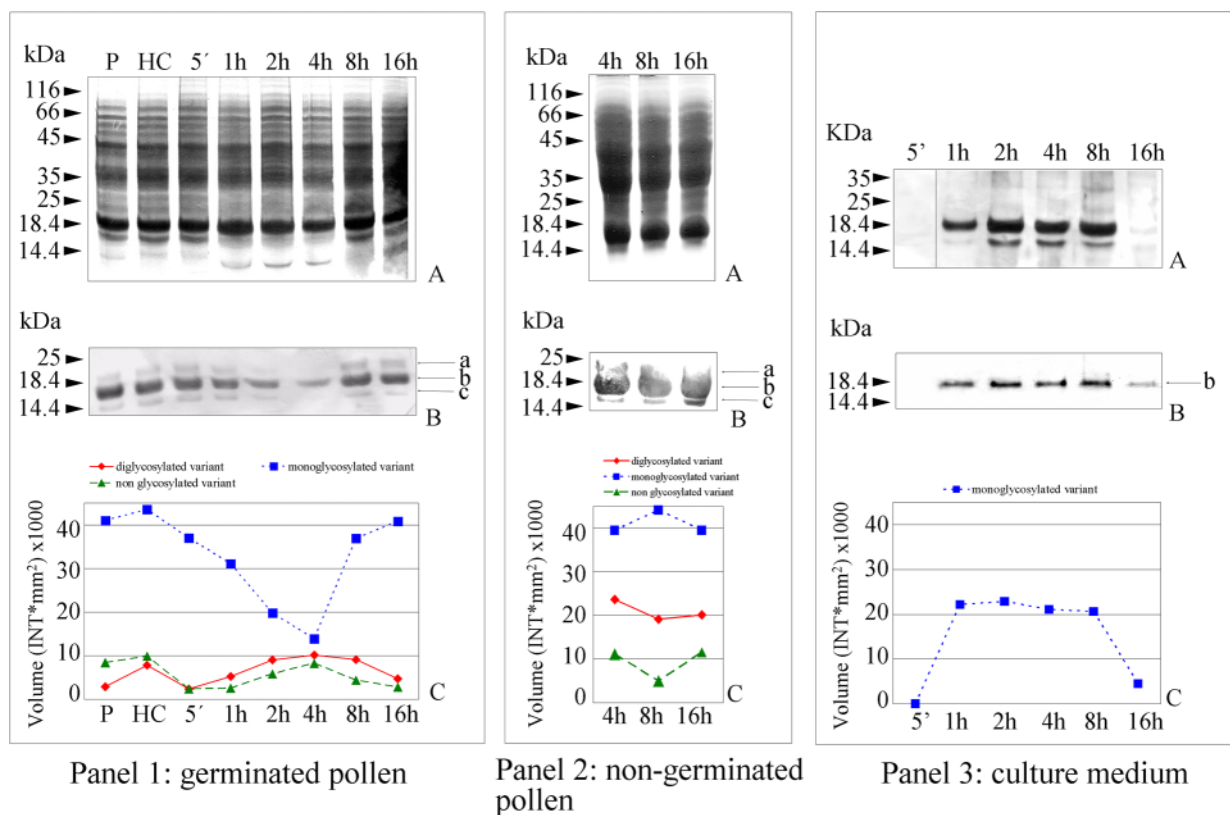


**Fig. 1** Expression of Ole e 1 in olive pollen during in vitro germination I. (Panel 1) germinated pollen samples; (Panel 2) non-germinated pollen samples; and (Panel 3) culture medium. From top to bottom: (A) Coomassie blue stained (silver for Panel 3) SDS-PAGE gels corresponding to crude protein extracts; (B) immunoblot of equivalent SDS-PAGE gels probed with the anti-Ole e 1 monoclonal antibody; (C) densitometry analysis of the antibody-reacting bands. P, mature pollen; HC, pollen after pre-hydration treatment; a and b, diglycosylated and monoglycosylated variants of Ole e 1 respectively; c, non-glycosylated variant.

interaction between LAT52 and the extracellular domain of the pollen receptor kinase LePRK2 (Tang et al. 2002).

This paper analyzes the temporal and spatial presence of Ole e 1 and its transcripts during in vitro olive pollen germination. We also discuss the possible coincidences between our findings and the putative role proposed for the Ole e 1-homologue LAT52.

## Results

### Olive pollen in vitro germination

Evidence of the initiation of olive pollen germination was detected approximately 1 h after culture onset in the form of light swelling of the pollen grains. Grains here adopted a spheroidal shape rather than the prolate shape typical of this pollen. Pollen tube emergence was detected after approximately 1 h of culture. However, only following 2 h incubation did effective separation of non-germinated and germinated pollen grains become feasible by filtration. Tube growth persisted over 4, 8 and even 16 h of in vitro culture, reaching lengths of higher than 20 times (600 µm) pollen diameter. After 16 h no further progress in pollen tube elongation was detected. Nevertheless,

evidence of degeneration, breakage of pollen tubes and contamination of the cultures were observed. Total percentages of pollen germination ranged between 30 and 50% mainly depending upon the pollen storage conditions.

### Ole e 1 expression analysis during in vitro olive pollen germination

Sections A of Fig. 1 illustrate the evolution of polypeptide profiles of crude protein extracts from mature and in vitro germinated olive pollen (panel 1), non-germinated pollen (panel 2) and the culture medium (panel 3) following SDS-PAGE and Coomassie blue staining (silver staining for panel 3). Patterns in panel 1 display the presence of numerous bands and were similar for all analyzed samples. One group of proteins in the range of 18–20 kDa was the most prominent. As compared to the mature or the chamber-hydrated pollen (panel 1) no conspicuous changes in either the band pattern or their relative intensity were detected in the pollen germinated for 5 min, 1, 2, 4, 8 and 16 h. The 18–20 kDa polypeptides were the only exception, their intensity slightly diminishing between 1 and 4 h of culture and finally rising again to the former level as of 8 h. Protein profiles of non-germinated pollen after 4, 8

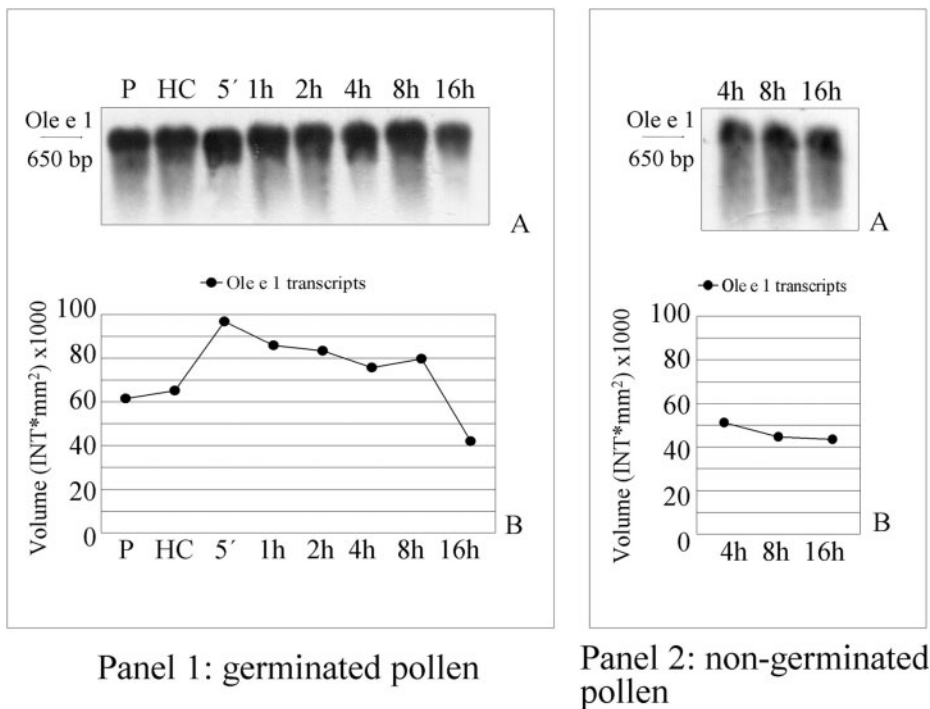


**Fig. 2** Expression of Ole e 1 in olive pollen during in vitro germination II. (Panel 1) germinated pollen samples; (Panel 2) non-germinated pollen samples. From top to bottom: (A) Northern blot analysis of Ole e 1 transcripts; Ole e 1 DNA probe hybridized to an abundant single band of approximately 650 bp in all of the samples analyzed; Loading: 5 µg of total RNA per lane on 2% denaturing agarose gels. (B) Densitometry analysis of the hybridizing bands. P, mature pollen; HC, pollen after pre-hydration treatment.

and 16 h of culture (panel 2) were also similar to those of panel 1, showing no significant changes in the intensity of the 18–20 kDa bands. Silver staining of the gels corresponding to the culture medium displayed a major protein species of ca. 20 kDa. This protein could not be detected in the sample obtained at only 5 min after the onset of the culture. The intensity of this band remained constant between 1 and 8 h of culture, but became significantly lower in the sample corresponding to 16 h culture.

Sections B of Fig. 1 represent immunoblots of the same gels in the corresponding A sections after probing with anti-Ole e 1 monoclonal antibody. Three reactive bands can be distinguished in panels 1 and 2 matching the molecular masses of the non-glycosylated (18.4 kDa), the glycosylated (20 kDa) and the diglycosylated (22 kDa) variants of Ole e 1. Only the glycosylated variant of the protein was detected in the immunoblots corresponding to samples from the culture medium (panel 3).

A densitometry analysis of the relative density of each immunoreactive band is displayed in the C sections of Fig. 1.

The levels of Ole e 1 transcripts during in vitro germination were analyzed by Northern blot. In all of the analyzed samples Ole e 1 DNA probe hybridized to an abundant single band of approximately 650 bp (Fig. 2). During pollen germination (Panel 1), the levels of Ole e 1 transcript remained relatively high and similar to those of the mature pollen samples, with the exception of a slight increase 2 h after the onset of the culture, and a significant decrease at the very end of the culture (16 h). The non-germinated pollen grains also showed high transcript levels. These levels underwent a slight decrease over the entire culture period.

#### *Immunolocalization of Ole e 1 in germinated olive pollen*

Fig. 3 illustrates Ole e 1 immunolocalization by fluorescence microscopy. Positive labeling appeared as a greenish fluorescence in comparison to the natural yellowish autofluorescence. Labeling first occurred in the three aperture regions of the olive pollen after 1–2 h incubation, regardless of which aperture region finally produced a pollen tube (Fig. 3A). After emergence of the pollen tube, the fluorescent signal was detected in the subapical and apical regions (Fig. 3B, C). Fluorescent deposits were frequently stacked on the outside of the pollen tube after 2–8 h of culture (Fig. 3C, D). This fluorescence was not, however, associated with the presence of materials or structures visible by light microscopy. The occurrence of such deposits was highly dependent on the extension of the washing steps during the immunolocalization procedure. Thus they were not distinguishable with the application of intensive washes (not shown). This subapical and apical location of Ole e 1 remained the same throughout pollen tube growth independently of its length. No fluorescence was detected in proximal regions of the tube, in the callose plugs (Fig. 3D) or in the negative controls prepared by omitting the primary antibody from the immunodetection procedure (Fig. 3E).

At the transmission electron microscopy level, the subapical region of the pollen tube displays an organelle-rich cytoplasm in which mitochondria, plastids, dictyosomes and a large amount of lipids and endoplasmic reticulum cisternae are observable (Fig. 4A). The pollen tube cell wall is composed of an electron-dense outer layer and an electron-clear inner layer. Gold labeling was detected following immunolocalization in the lumen of RER cisternae, scattered throughout the cyto-

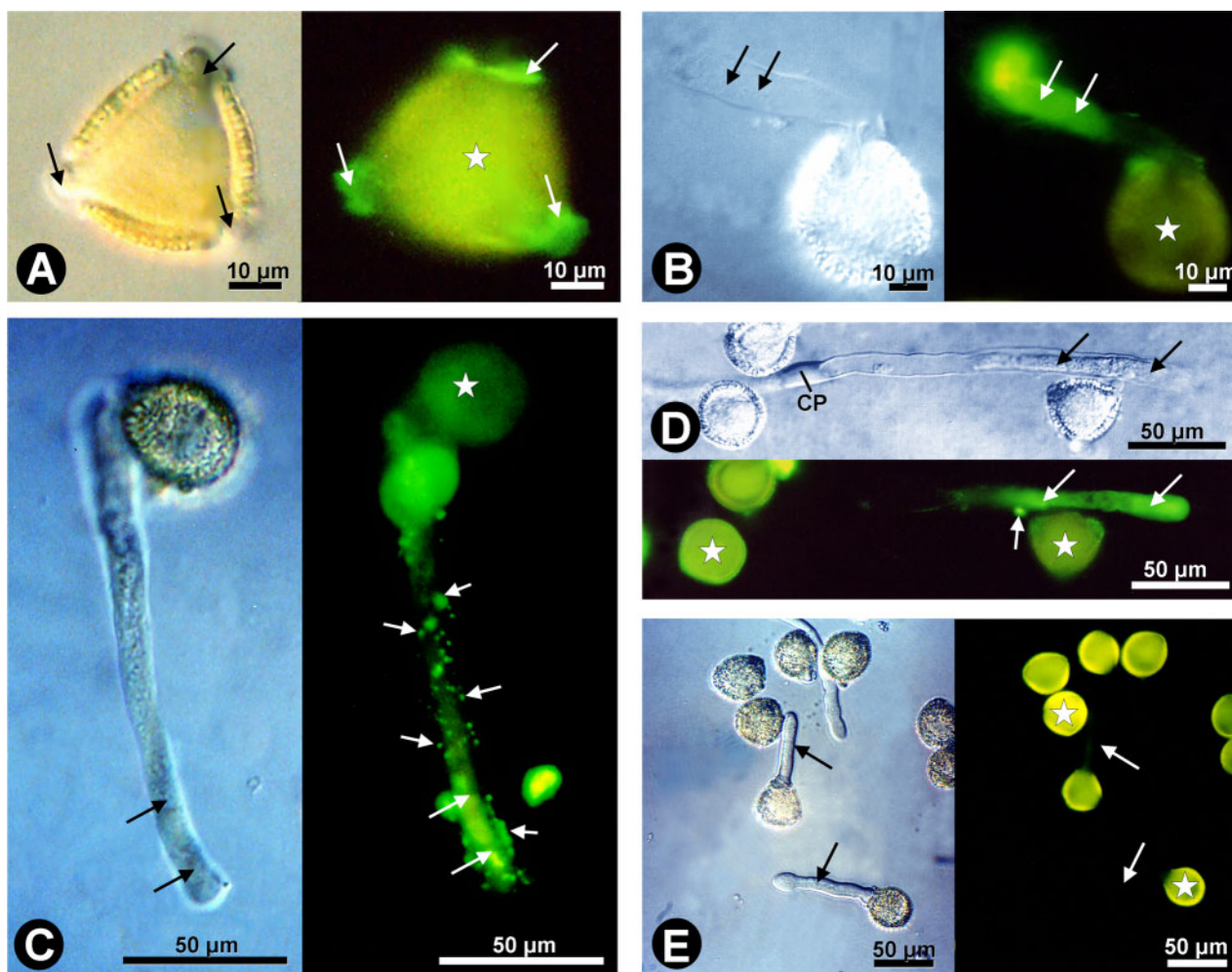


**Fig. 3** Localization of Ole e 1 in the germinating pollen grain by immunofluorescence. Figures include a differential interference contrast (DIC) reference image and the corresponding image obtained by epifluorescence. (A) Greenish fluorescence showing the presence of the allergen in the three aperture regions (arrows) after 2 h of in vitro culture. (B–D) Fluorescent signal (arrows) detected in the subapical and apical regions of the emerged pollen tube after 4, 8 and 16 h incubation, respectively. Fluorescent deposits are stacked on the outside of the pollen tube (small arrows). (E) Negative control prepared by omitting the primary antibody from the immunodetection procedure. CP, callose plug; stars, yellowish autofluorescence of the pollen wall.

plasm (Fig. 4B). Lipids and organelles such as mitochondria and plastids were devoid of gold particles. Extracellular labeling was also detectable outside the pollen tube. Negative controls prepared by omitting the primary antibody showed no significant labeling over the background (Fig. 4C).

### Discussion

In vitro pollen germination is able to mimic the in vivo cellular processes. Therefore, within a complex cellular system like the style, it has been used as an alternative method to overcome the difficulties inherent to these studies, such as the reduced period of pollination and the performance of time-lapse studies (particularly at the biochemical/molecular level). Using this in vitro system, a number of studies have recently

described those developmental changes concerning the ultra-structure, the cytoskeleton, nuclei mobility and the behavior of storage lipids which occur in the olive pollen during germination (Rodríguez-García et al. 2003a, Rodríguez-García et al. 2003b). The experimental system described in the present paper efficiently allows germinated and non-germinated pollen grains to be separated, and is thus a useful tool particularly for the analysis of the molecules involved in these processes.

#### *Ole e 1 allergen increases its expression and is released into the aqueous medium during in vitro olive pollen germination*

The present study confirms the presence of a significant increase in the proportion of this protein during the course of germination, in comparison to the non-germinated pollen, in which the level of Ole e 1 protein remains constant. Our obser-

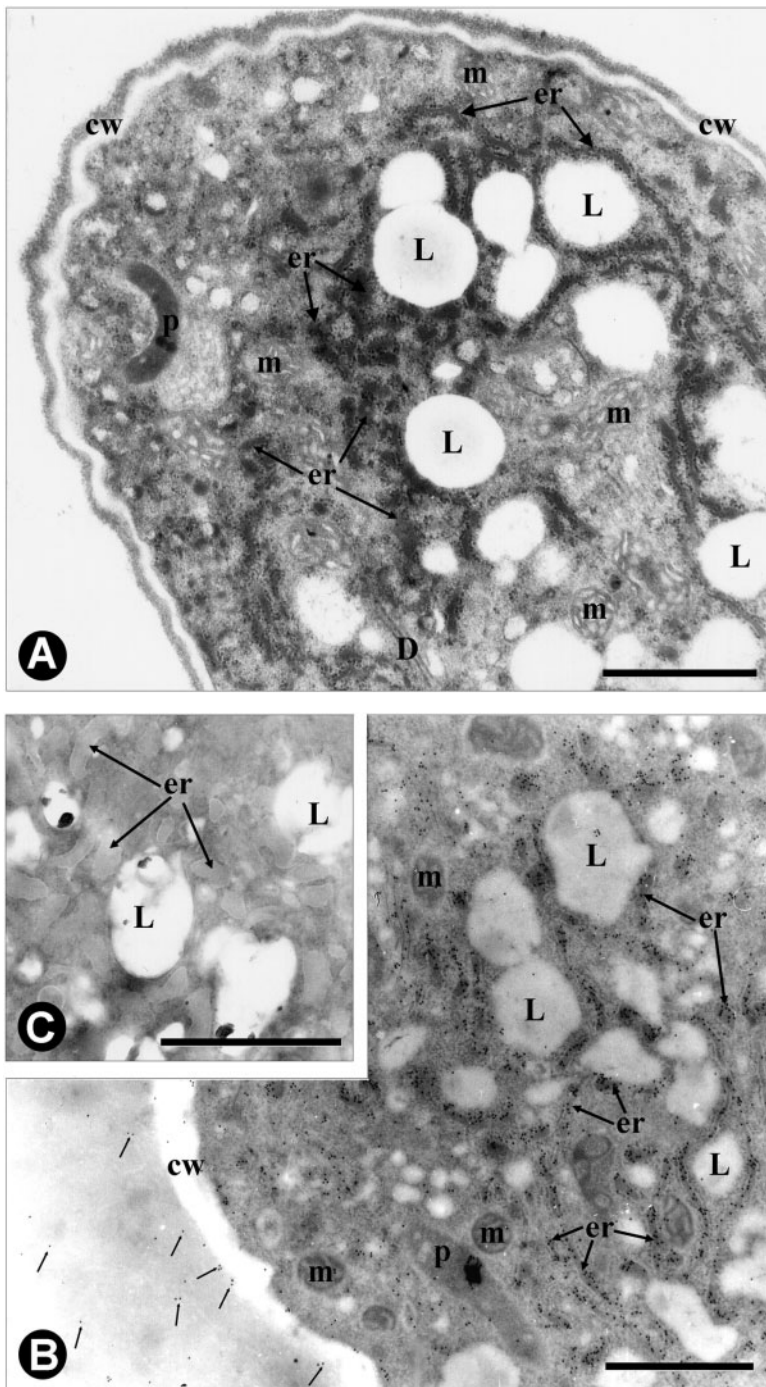


**Fig. 4** TEM Immunolocalization of Ole e 1 in the olive pollen tube during in vitro germination. (A) TEM overview of the subapical region of a pollen tube germinated for 4 h. (B) Immunolocalization of Ole e 1: gold particles decorate the endoplasmic reticulum cisternae. Some labeling is also detected outside, although in the vicinity of the pollen tube (small arrows). (C) Negative control prepared by omitting the primary antibody. Cw, cell wall; D, dictyosomes; er, endoplasmic reticulum; L, lipids; m, mitochondria; p, plastid. Bars represent 1  $\mu$ m.

vations may resemble those of Muschiatti et al. (1994) regarding the Ole e 1 homolog LAT52. Antisense repression experiments carried out by these authors using *LAT52* complementary transcripts produced abnormal functioning in tomato pollen, including hydration and/or germination deficiencies. In the present study, the naturally occurring non-germinated pollen displayed lower levels of both Ole e 1 and Ole e 1 transcripts than the corresponding samples of germinated pollen. If

a procedure to separate both pollen categories were to be developed for samples obtained at 5 min, 1 and 2 h after the onset of the culture, the differences among germinated and non-germinated pollen may well be even more conspicuous.

Ole e 1 was found in the form of three variants (non-glycosylated, mono and diglycosylated), as has been extensively described in the mature olive pollen grain (Lauzurica et al. 1988a, Lauzurica et al. 1988b, Villalba et al. 1990, Batanero

et al. 1994b) and during the late stages of olive pollen development (Alché et al. 1999). The high degree of polymorphism detected for this protein is also a characteristic for many other pollen allergens (Johnson and Marsh 1965, Bond et al. 1991, Griffith et al. 1991, Swoboda et al. 1995a). No significant changes were observed in the relative proportion of these three variants. The monoglycosylated variant was the most abundant, and therefore the only one detected in those samples where Ole e 1 was scarce (e.g., in the culture medium). The appearance of newly synthesized glycosylated forms of Ole e 1 between 4 and 8 h of the culture, parallel to the increase in the levels of the non-glycosylated variant, indicate that glycosylation mechanisms are probably present and active in the pollen tube cytoplasm.

Several works have reported the allergen gene expression patterns in pollen during anther development in birch (Swoboda et al. 1995b), rice (Xu et al. 1995), barley (Astwood and Hill 1996), *Brassica rapa* (Okada et al. 1999) and olive (Alché et al. 1999). However, until now no data have been available concerning the expression pattern of these genes during pollen germination. Our paper shows that Ole e 1 transcripts are massively present, not only in the mature pollen grain, but also during in vitro pollen germination. The fact that high levels of the allergen coincide with this massive presence of transcripts also confirms the initial hypothesis according to which Ole e 1 expression is controlled at the transcriptional level (Alché et al. 1999). Similar results were found when *NTP-303* gene expression was analyzed in tobacco (Weterings et al. 1992), in which transcripts accumulated in the apex region of the pollen tube. On the contrary, no significant changes were reported for the *BCP-1* gene expression analysis in the pollen of *Brassica campestris* (Theerakulpisut et al. 1991) nor for several mRNAs from *Petunia inflata* pollen (Mu et al. 1994a, Mu et al. 1994b) during germination. The delay in the appearance of the newly synthesized protein (between 4 and 8 h of culture) with respect to the short peak in the presence of transcripts (immediately after pollen hydration and activation) was also observed during several stages of olive pollen development (Alché et al. 1999). This delay was attributed to the particular cycle of rRNA during pollen development, more than to the existence of a post-transcriptional regulatory mechanism.

In most plant species, both the late growth of pollen tube and the division of the generative cell depend upon a new synthesis of transcripts within the pollen tube, once germination has begun (Mascarenhas 1975). The increase in Ole e 1 transcript levels detected 2 h after the onset of the germination supports these data. In contrast, pioneering studies in the use of RNA synthesis inhibitors, such as actinomycin D, suggest that both pollen germination and the early growth of pollen tube are not dependent on de novo synthesized transcripts, since mRNAs, ribosomes and tRNAs accumulate during pollen maturation (Mascarenhas 1988, Mascarenhas 1993). Several studies carried out in *Tradescantia* (Mascarenhas et al. 1974,

Mascarenhas and Mermelstein 1981), maize (Hanson et al. 1989) and, more recently, in tobacco (Weterings et al. 1992), reported non-significant qualitative differences between the most abundant and representative transcripts in the mature pollen grain prior to germination, and those de novo mRNAs synthesized during germination. The results presented here confirm that Ole e 1 transcripts are found in all tested samples and that new synthesis only occurs once germination has further progressed. As previously demonstrated throughout olive pollen development (Rodríguez-García et al. 1995, Alché et al. 1999), the endoplasmic reticulum cisternae have also been recognized as the place in which Ole e 1 synthesis and storage occur during pollen tube growth.

Finally, the expulsion of respirable allergen-containing submicronic materials has been proposed as one of two accepted mechanisms underlying allergen release to the human mucosal surfaces and deeper airways in grasses (Grote et al. 2001). These submicronic materials are mainly composed of starch granules in ryegrass pollen and the denominated polysaccharide particles (P-particles) in timothy grass pollen. In the mature olive pollen, a number of studies have reported the absence of such structures (Rodríguez-García et al. 2003a), as well as the localization of the allergen Ole e 1 in association with ER cisternae and the pollen wall (Rodríguez-García et al. 1995, Alché et al. 1999). Therefore, the involvement of a mechanism similar to that of rapid allergen diffusion in isotonic media (e.g., nasal secretions) widely demonstrated for many allergens and pollens (Vrtala et al. 1993) is proposed for Ole e 1 release. A recent study (Carnés et al. 2002) has verified that Ole e 1 is rapidly released from the pollen grain with different kinetics after extraction in deionized water and several solvents. The detection of Ole e 1 in the culture medium during in vitro germination, as well as its location close to the pollen tube wall, as reported in the present work, also account for the presence of such diffusion mechanisms. Based on our observations, we propose at least three different pathways for this Ole e 1 release: (1) directly from the pollen wall, where the allergen is particularly abundant; (2) through the aperture regions of the pollen upon pollen hydration, regardless of whether the pollen grain does or does not germinate; and (3) through the pollen tube cell wall during pollen tube growth. Rapid elution or solubility is considered an important prerequisite for a protein to behave as a major allergen (Gupta et al. 1995, Grote et al. 2001, Carnés et al. 2002), in addition to the biological implications that it may have for the allergen function within the pollen grain.

#### *Ole e 1 as an extracellular signaling molecule: a new prospect*

In spite of evidence that Ole e 1 may be a key molecule for pollen hydration and pollen tube growth, little information is available concerning the molecular mechanisms underlying this function. We have previously suggested (Alché et al. 1999) a putative role for this allergen in the creation of the osmotic gradient needed for pollen hydration and germination,

a mechanism that was already proposed by Heslop-Harrison and Shivanna (1977). The results obtained in this paper show a high level of Ole e 1 expression throughout in vitro pollen germination. Such expression may contribute to the maintenance of this osmotic gradient in the pollen tube. However, we have recently detected that major differences exist in the Ole e 1 content (ranging from 3 to 23% of the total protein weight) between olive cultivars (Castro et al. 2003), although Ole e 1 is present in the pollen of all varieties. Experiments are currently in progress to determine whether significant changes among cultivars can be detected in the capacity of pollen to hydrate and/or germinate.

According to a second hypothesis, the protein would be involved in recognition phenomena between pollen-stigma and pollen tube-style cells, as suggested by the presence of the allergen in the pollen exine, the pollen tube and the germination medium. Recent discoveries (Tang et al. 2002, Wengier et al. 2003) show that extracellular LAT52 interacts with two tomato pollen receptor kinases (LePRK1 and principally LePRK2). This interaction might be an autocrine signalling system which regulates the initiation, maintenance and even the guidance of pollen-tube growth, thus enabling successful fertilization. The model proposed by these authors involves a pregermination association of LAT52 to the receptor kinases. Upon germination on the stigma, the associated LAT52 would be displaced by a style component and the induction of LePRK2 dephosphorylation would trigger a signal transduction cascade to the pollen tube.

LAT52 shares many features with Ole e 1, including its cysteine-rich and glycosylated character, its relatively high sequence homology, and also its secretory nature. Both proteins are equally heat-stable, display similar apparent molecular mass after SDS-PAGE, and are massively expressed in the mature pollen. The LAT52/LePRK1-LePRK2 signaling model may well apply to Ole e 1 and many other homologous proteins characterized in the pollen of different plant species. However the olive pollen homologs of the LePRK1 and LePRK2 receptor kinases have yet to be characterized. The in vitro germination system as described in the present study will help to understand whether the early pregermination steps of the model are also shared by the Ole e 1 function. Future studies of in vivo germination and/or experiments including the addition of stylar extracts to the in vitro germination system will further clarify whether the putative Ole e 1 displacement does in fact occur.

## Materials and Methods

### *In vitro pollen germination*

Mature pollen grains taken from selected *Olea europaea* L. trees (cv. Picual) in Granada (Spain) were collected during the anthesis period using large paper bags by vigorously shaking the inflorescences. Pollen was sequentially sieved through mesh (pore sizes of 150 and 50  $\mu$ m) in order to separate pollen grains from debris. Grains were then frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ .

Pre-hydration of stored pollen was performed by incubation in a humid chamber at room temperature for 30 min. Pollen (1 g/plate) was then transferred to Petri dishes containing 10 ml of germination medium [10% (w/v) sucrose, 0.03% (w/v)  $\text{Ca}(\text{NO}_3)_2$ , 0.01% (w/v)  $\text{KNO}_3$ , 0.02% (w/v)  $\text{MgSO}_4$  and 0.03% (w/v) boric acid], as described by M'rani-Alaoui (2000). Petri dishes were maintained at room temperature in the dark and pollen samples were taken at 5 min, 1, 2, 4, 8 and 16 h following onset of the culture (Fig. 1). Germinated and non-germinated pollen grains from each sample were separated by filtration through a 50  $\mu$ m mesh and finally pelleted by centrifugation (5,000 $\times$ g for 10 min). The supernatant (culture medium) was kept for SDS-PAGE analysis.

### *Protein extraction and SDS-PAGE and immunoblot analysis.*

Pollen samples were ground in a  $\text{IN}_2$ -precooled mortar to a fine powder, re-suspended in an extraction buffer [50 mM Tris-HCl (pH 7.5), 0.5 mM ethylene glycol-bis( $\beta$ -aminoethyl ether) (EGTA), 5 mM ascorbic acid, 100 mM dithiothreitol (DTT) and 2 mM phenylmethylsulfonyl fluoride (PMSF)] to a proportion of 10 ml solution per g of fresh tissue, and then incubated on ice for 30 min with occasional shaking. After centrifugation at 14,000 $\times$ g at  $4^{\circ}\text{C}$  for 20 min, supernatants were stored at  $-20^{\circ}\text{C}$ . Protein concentration in each sample was measured following the Bradford (1976) method [Bio-Rad reagent and bovine serum albumin (BSA) as standard].

Samples prepared as above (30  $\mu$ g protein each) and 30  $\mu$ l samples of the germination medium at different times of incubation were loaded into a 12% sodium dodecyl sulfate (SDS)-acrylamide gel as described by Laemmli (1970). Proteins were separated using a Mini-Protein II system (Bio-Rad, U.S.A.). Gels were stained with Coomassie blue or silver, or they were transferred onto polyvinylidene difluoride (PVDF) membranes at 100 V for 1.5 h in a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad, U.S.A.). For immunodetection, after incubation with a blocking solution containing 0.1% (v/v) Tween 20 and 3% (w/v) skimmed dried milk in Tris-buffered saline (TBS), membranes were probed with a monoclonal antibody to Ole e 1 (Lauzurica et al. 1988a) diluted 1/1,000 in the same buffer. The secondary antibody was a rabbit anti-mouse IgG-alkaline phosphatase conjugated antibody (Promega Corporation, U.S.A.) diluted 1/2,500 in TBS. Detection was carried out by using 4-nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) (Amresco, U.S.A.) as the substrate for the enzyme. Densitometry analysis of the relative amount of Ole e 1 within the pollen grain and in the culture medium during in vitro germination was performed using the Quantity One 4.2.0 (Bio-Rad, U.S.A.) software.

### *Total RNA extraction and Northern blot analysis*

Total RNA from germinated pollen was extracted at different times of germination by using an RNeasy Plant Total RNA kit (Quiagen, U.S.A.). Samples were ground in a mortar with liquid  $\text{N}_2$  to a fine powder, and processed according to the manufacturer's instructions. Both the quality and the concentration of total RNA were assessed by denaturing gel electrophoresis and calculating  $A_{260}/A_{280}$ . Samples were immediately processed for Northern blot analysis.

Five  $\mu$ g of total RNA of each sample was separated in 2% (w/v) agarose denaturing gels according to Jones (1995) and then transferred onto Biodyne Plus membranes (Pall BioSupport, U.S.A.) following the protocol described by Sambrook et al. (1989). Equivalent loading for all lanes was assessed by methylene blue staining of blots and quantification of rRNA bands. Membranes were then hybridized according to the instructions of "The DIG System User's Guide for Filter Hybridization" (Boehringer Mannheim GmbH, Germany) using a dig-labeled DNA probe. The probe was prepared by PCR amplification of an Ole e 1 cDNA (clone 3c: 438 bp) (Villalba et al. 1994),

inserted in a pGEM-4Z plasmid (Promega Corporation, U.S.A.). PCR was performed as described by Leitch et al. (1994) using T7 and SP6 as primers. The amplified fragment was ethanol-precipitated (Sambrook et al. 1989), checked by agarose gel electrophoresis and finally, quantified by measuring  $A_{260}/A_{280}$ .

Hybridizing bands were detected with an anti-digoxigenin alkaline phosphatase conjugated antibody (Fab), diluted 1/10,000, and disodium-2-chloro-5-(4-methoxyspiro{1,2-dioxetane-3,2'-(5'-chloro)tricyclo[3.3.1.1<sup>3,7</sup>]decan}-4-yl)-1-phenylphosphate (CPD-Star) as the chemiluminescent substrate (both chemicals from Boehringer-Mannheim GmbH, Germany). Hyperfilm ECL film (Amersham, U.K.) was used to register the signal.

#### Fluorescence microscopy (FM) and transmission electron microscopy (TEM) immunolocalization analysis

For FM studies, in vitro germinated pollen samples were transferred to 1.5 ml disposable plastic tubes and then fixed at 4°C for 30 min in a solution containing 4% (w/v) paraformaldehyde in BSM buffer [50 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid); 1,4-piperazinediethanesulfonic acid (PIPES) (pH 6.8), 1 mM MgSO<sub>4</sub>·7H<sub>2</sub>O and 5 mM EGTA]. Samples were subsequently incubated at 4°C in the following solutions for the times indicated, using gentle agitation: blocking solution [5% (w/v) BSA, 1% (v/v) Triton X-100 prepared in BSM buffer] 10 min; washing solution [1% (v/v) Triton X-100 in BSM buffer], three changes of 10 min each; anti-Ole e 1 monoclonal antibody [diluted 1/200 in blocking solution] overnight; washing buffer as above; goat anti-mouse IgG fluorescein isothiocyanate (FITC)-conjugated secondary antibody (Sigma, U.S.A.), diluted 1/1,000 in blocking solution, 2 h in the dark; and final rinses in washing buffer. At the end of each incubation step, pollen grains were allowed to settle by gravity and then the corresponding solution replaced by the next. Finally, samples were re-suspended in a CITIFLUOR/Glycerol/Phosphate-buffered saline (PBS) antifading solution (Sigma, U.S.A.) and observed in a Zeiss Axioplan photomicroscope equipped with an epifluorescence system, under blue light irradiation. Images were registered in 100 ASA Kodak color films. Negative controls were treated as described above, but the primary antibody was omitted.

For TEM studies, samples were processed as previously reported (Rodríguez-García et al. 1995) with minor modifications. Germinated pollen grains were isolated from the germination medium as described above and fixed for 2 h at room temperature in a solution of 2.5% (v/v) glutaraldehyde and 2% (w/v) paraformaldehyde in 0.1 M cacodylate buffer (pH 7.2). Samples were dehydrated in an ethanol series, gradually transferred to propylene oxide and embedded in Epon. Ultrathin sections (80 nm) were obtained with a Reichert-Jung ultramicrotome and transferred onto 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 1.5 h with the same anti-Ole e 1 antibody, diluted 1/20 in blocking solution. After washing several times with PBS the grids were treated for 2 h with a goat anti-rabbit IgG secondary antibody coupled to 20 nm gold particles (Biocell International, U.K.) diluted 1/30 in PBS. Finally, they were washed in PBS, rinsed in double-distilled water and then stained for 15 min with a solution of 5% (w/v) uranyl acetate. Observations were carried out in a Zeiss EM10C transmission electron microscope. Treatment of control sections was the same, although incubation with the primary antibody was omitted.

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