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TEMPORAL AND SPATIAL GENE EXPRESSION OF Ole e 3 ALLERGEN IN OLIVE (*OLEA EUROPAEA* L.) POLLEN

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This paper describes the distribution of Ole e 3 allergen and its transcripts in the developing anther and in mature olive pollen. Northern blot and RT-PCR analyses over the course of pollen development show that Ole e 3 transcripts accumulate exclusively in mature pollen. This gene therefore corresponds to a "late gene." The sequences amplified by RT-PCR display high identity with those already reported for Ole e 3, including two Ca^{2+}-binding motifs. Immunoblot analysis of the allergen shows that Ole e 3 accumulates during the same stage as the corresponding transcripts, suggesting a transcriptional regulation mechanism for the expression of the gene. Finally, the use of transmission electron microscopy techniques has shown that (a) the allergen is located mainly in the vicinity of membrane systems and in the aperture regions of the mature pollen grain, and (b) Ole e 3 transcripts are detectable after using in situ RT-PCR. These data are significant clues to the biological roles of the protein in olive pollen biology. Such putative functions are discussed.

Key words: *Olea europaea* L., allergen, expression, localization, Ole e 3, olive.

INTRODUCTION

Olive tree pollen is a widespread cause of respiratory allergy in the Mediterranean basin. Ten of the protein allergens present in this pollen have been characterized, and important evidence for the biological functions of several has been reported (Huecas et al., 2001; Rodríguez et al., 2001). However, a detailed study of their expression during pollen development has only been described for the major allergen Ole e 1 (Alché et al., 1999). In this paper we describe the spatial and temporal expression of the Ole e 3 allergen. The later is a 9.2 kDa polypeptide exhibiting two 12-residue segments homologous to Ca^{2+}-binding sites of the EF-hand type. Ca^{2+}- binding capacity has been demonstrated for both the natural and the in vitro-expressed allergen (Ledesma et al., 1998). These authors also demonstrated the presence of Ole e 3 homologous proteins in mature pollen of a variety of species related and unrelated to the olive tree, and they suggested the tentative generic name "polcalcins" for these Ca^{2+}-binding proteins.

MATERIALS AND METHODS

PLANT MATERIALS

Floral buds and leaves from selected *Olea europaea* L. trees (cv. Picual) in Granada (Spain) were collected between April and June. After LM staging of flowers, anthers were dissected out and immediately frozen in liquid nitrogen. Dehiscent pollen was collected in bags by vigorously shaking the inflorescences, and then sieved to remove debris.

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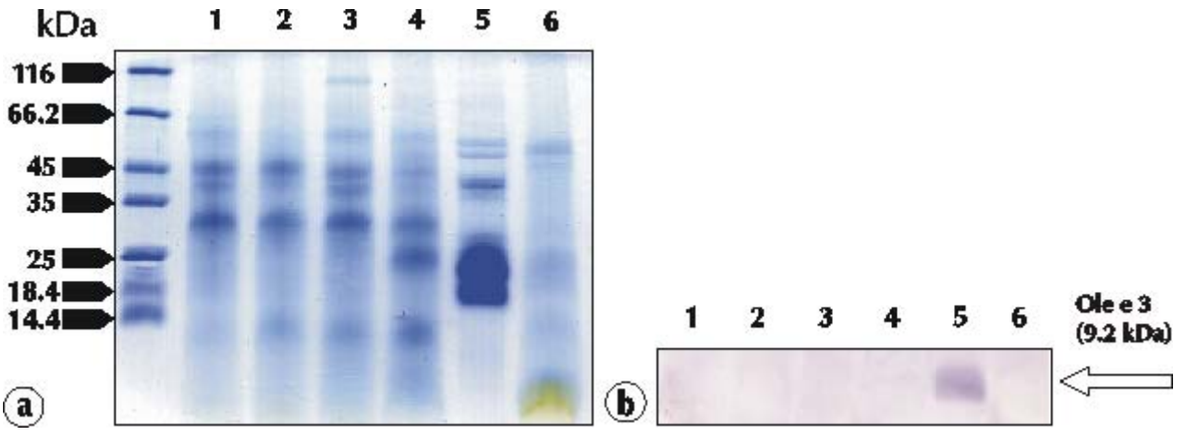

Fig. 1. (**a**) Coomassie-stained 12% Tricine-SDS-PAGE gel corresponding to protein crude extracts obtained from anthers at different stages and dehiscent pollen. Lane 1 – tetrads; Lane 2 – young microspores; Lane 3 – vacuolated microspores; Lane 4 – early pollen within the anther; Lane 5 – dehiscent pollen; Lane 6 – olive leaves (control), (**b**) Immunoblot of a gel as in (a), probed with Ole e 3 polyclonal serum. 30 µg total protein was applied to each lane.

PREPARATION OF PROTEIN EXTRACTS AND IMMUNOBLOTING

The procedures used here are extensively described in Alché et al. (1999). For immunodetection, the membranes were probed with a polyclonal antibody to Ole e 3 (Ledesma et al., 1998), diluted 1/200. Goat anti-rabbit IgG-alkaline phosphatase conjugate (Sigma) diluted 1:5000 was used as the secondary antibody, and color was developed with NBT/BCIP.

REVERSE TRANSCRIPTION-POLYMERASE CHAIN (RT-PCR) ANALYSIS

RNA isolation, cDNA synthesis and PCR were carried out as described for Ole e 1 analysis (Alché et al., 1999). The oligonucleotide primers for PCR were designed based on the nucleotide sequence of Ole e 3 (Ledesma et al., 1998): Ole e 3 (sense) primer (5'-CCGACGATCCACAGGAAGTAGC-3') and Ole e 3 (antisense) primer (5'-CTTGGCAACGTCCTTGACTAATCC-3'). PCR amplification was performed by 20 cycles at 94˚C for 1 min, at 66˚C for 1 min 30 s and at 72˚C for 1 min.

IMMUNOLOCALIZATION

Unicryl-embedded samples and ultrathin sections were prepared as described in Alché et al. (1999). Nonspecific binding sites were blocked by incubation of the sections in 5% BSA, 1% normal goat serum in PBS for 15 min. This was followed by treatment with anti-Ole e 3 polyclonal antiserum diluted 1:10 in blocking reagent for 1 h. The grids were treated with goat anti-rabbit IgG coupled to 10 nm gold particles (BioCell International) diluted 1:50 in PBS for 45 min. After washing, sections were stained for 10 min in 5% uranyl acetate and observed. Negative sections were treated as described, but only blocking reagent instead of the anti-Ole e 3 antibody solution was used to incubate the grids.

IN SITU REVERSE TRANSCRIPTION PCR LOCALIZATION OF Ole e 3 TRANSCRIPTS

IS-RT-PCR was performed basically as described in Alché et al. (2002). A poly-dT RACE adapter [5'-GACTCGAGTCGACATCGA$(T)_{17}$-3'] was used to carry out the RT phase, while Ole e 3 sense and antisense primers were used for the PCR amplification step.

RESULTS

Ole e 3 IS DETECTED EXCLUSIVELY IN MATURE POLLEN AFTER SDS-PAGE AND IMMUNOBLOT ANALYSIS

Figures 1a and b, respectively, show a Coomassie-stained SDS-PAGE gel and the results obtained after immunodetection of polypeptides by Ole e 3 polyclonal antiserum on electrophoresed protein

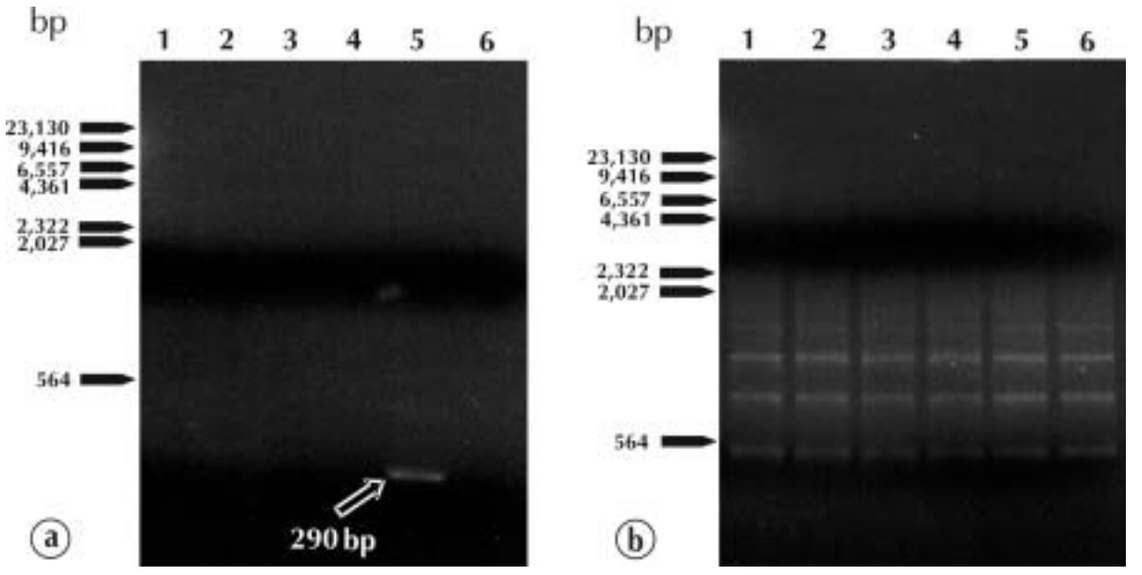

Fig. 2. (**a**) 1% TBE agarose gel showing RT-PCR amplification products corresponding to Ole e 3 transcripts at different developmental stages. Lane 1 – tetrads; Lane 2 – young microspores; Lane 3 – vacuolated microspores; Lane 4 – early pollen within the anther; Lane 5 – dehiscent pollen; Lane 6 – negative control (olive leaves), (**b**) 1% TBE agarose gel showing RT-PCR amplification products corresponding to ubiquitin monomer to polyubiquitin pentamers at the same stages as gel in (a).

crude extracts obtained from anthers at different developmental stages and dehiscent pollen. The antibody recognized a single band of ~9–10 kDa present exclusively in the mature (dehiscent) pollen stage. No bands were detected during earlier stages of anther development or in the control extract (leaves). The size of the reactive band is in good agreement with the described size of Ole e 3.

Ole e 3 TRANSCRIPTS ARE PRESENT EXCLUSIVELY IN MATURE POLLEN

The levels of Ole e 3 transcripts were analyzed by RT-PCR amplification of total RNA extracted from anthers at different developmental stages, as well as in mature (dehiscent) pollen of *Olea europaea* (Fig. 2a). No signals were detected in the samples corresponding to early developmental stages, nor in the control sample (leaves). On the contrary, a weak band of ~290 bp was detected after amplification of the RNA extracted from dehiscent pollen. Figure 2b shows a control reaction performed in order to ensure that RNA loading was equivalent for all samples, since the band intensities resulting from amplification of ubiquitin monomers to polyubiquitin pentamers were comparatively identical.

The BLASTN 2.1.1 (Altschul et al., 1997) sequence similarity search for this fragment in the nr base (Genebank+EMBL+DDBJ+PDB non-redundant sequences, not included in SET, STS, GSS or HTGS) resulted in significant alignment (94%) to the published sequence of Ole e 3 (Genebank accession number AF 015810) (Ledesma et al., 1998).

Ole e 3 IS LOCATED IN ASSOCIATION WITH MEMBRANE SYSTEMS AND IN THE APERTURE REGIONS

Immunolocalization of Ole e 3 in the mature pollen grain of the Picual variety shows that the allergen is associated mainly with membrane systems in the vegetative cell cytoplasm (Fig. 3a). These membrane systems include (i) endoplasmic reticulum cisternae, (ii) the membranes surrounding poorly defined structures that may correspond to lipid inclusions, vesicles or even organelles (Fig. 3b), and (iii) the nucleus envelope (Fig. 3c). Immunodetection also occurred in some areas of the exine and more abundantly in the apertural regions (Fig. 3a). Nuclei, organelles (mitochondria and plastids), vesicles, lipid bodies and the intine showed no significant levels of detection. No gold particles were observed in controls prepared by omission of the primary antibody (Fig. 3d).

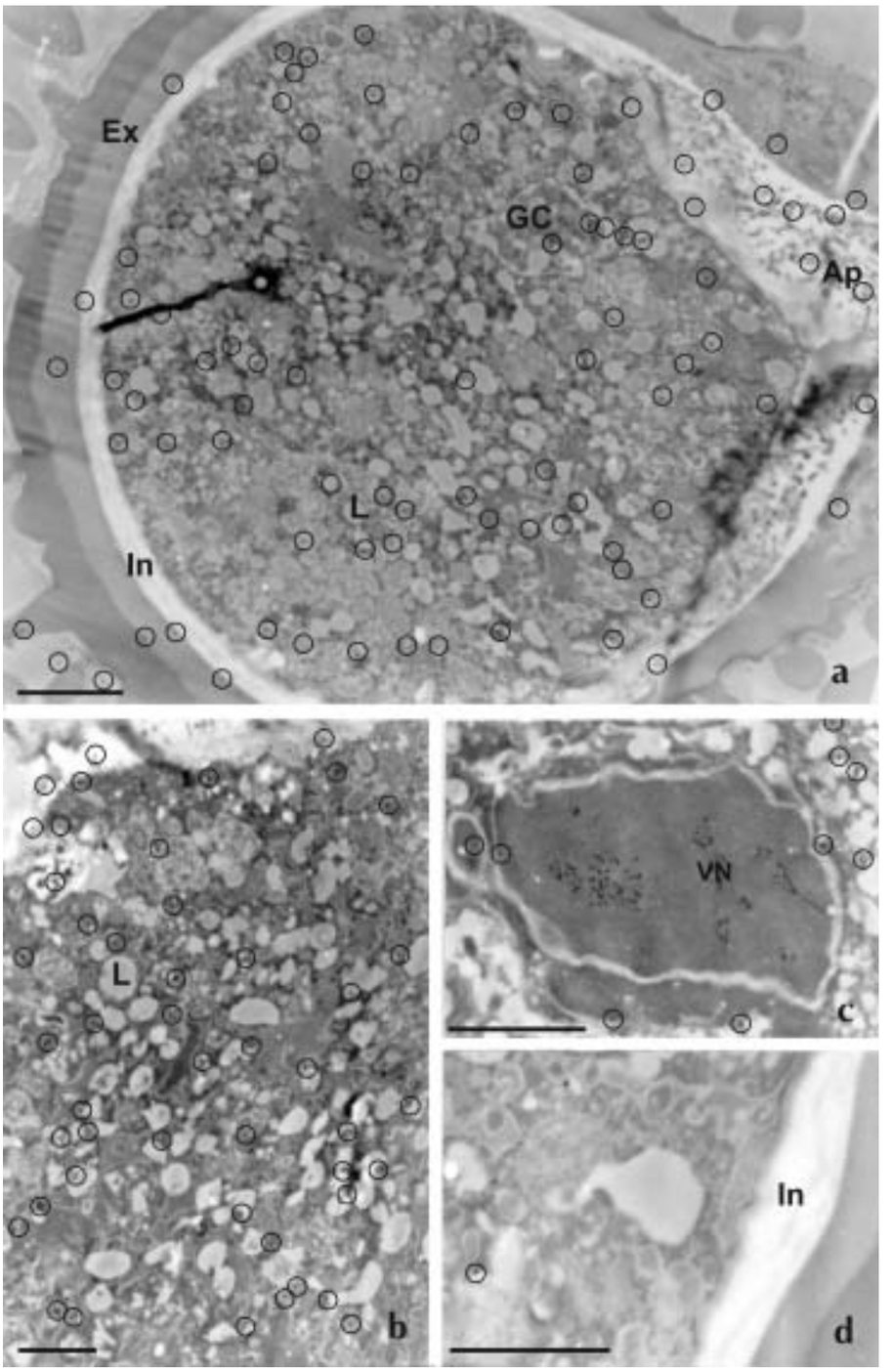

Fig. 3. Immunolocalization of Ole e 3 allergen in olive pollen. Gold particles are marked by circles. (**a**) General view of pollen grain. The allergen is associated largely to membrane systems in the vegetative cell cytoplasm. Labeling is also detected in exine regions and in the aperture, (**b**) Vegetative cell cytoplasm. Ole e 3 appears associated to ER membranes and poorly defined structures that may correspond to lipid bodies, vesicles and organelles. Organelles themselves, lipid bodies and intine show no labeling, (**c**) Vegetative cell nucleus displaying no gold particles, but in association with the nuclear envelope, (**d**) Negative control prepared by omitting the primary antiserum. Ap – aperture; Ex – exine; GC – generative cell; In – intine; L – lipids; VN – vegetative cell nucleus. Bar in all figures = 1 μm.

Ole e 3 TRANSCRIPTS ARE TEM-DETECTABLE IN MATURE POLLEN BY RT-IS-PCR

In situ reverse transcription followed by direct PCR enabled localization of Ole e 3 transcripts at the ultrastructural level in mature olive pollen (Fig. 4). Gold particles showing transcript location were found in the cytoplasm and nuclei of the vegetative and generative cells (Fig. 4a). Also, positive gold signaling was observed on condensed chromatin in vegetative and generative nuclei (Fig. 4b). No significant quantities of gold particles associated with vacuoles, lipid bodies or mitochondria were detected. Controls were performed with grids undergoing the same treatment, though reverse transcriptase enzyme was omitted in one case (Fig. 4c) and specific oligonucleotides in the other (results not shown). In both controls the presence of gold particles was restricted to a slight unspecific background.

DISCUSSION

Our results show that Ole e 3 is the product of one of the alleged "late genes" expressed only after the asymmetric division that originates the generative and vegetative cells (Twell, 1994). As compared with the expression of Ole e 1 – the only olive pollen allergen for which a detailed expression analysis has been performed (Alché et al., 1999) – Ole e 3 expression starts much later in the development process. Whereas Ole e 1 transcripts were already detectable within the anther at late tetrad stage, transcripts of Ole e 3 were only detected in the mature pollen stage. It can be inferred, therefore, that Ole e 3 expression probably occurs exclusively within the pollen grain and not in the tapetum (as in Ole e 1), because this tissue degenerates before the expression begins. Transcripts of other Ca^{2+}-binding pollen proteins such as Bra r 1 and Bra r 2 from *Brassica rapa* (Okada et al., 1999) have been detected in earlier stages of pollen development (e.g., the microspore), but those authors also found low levels of transcripts in the tapetum of the same species.

Ole e 3 has been defined as a member of the polcalcin family because of its Ca^{2+} ion-binding capacity and its specific presence in the pollen of several species (Ledesma et al., 1998). This may suggest that there is a direct relationship between the location of this allergen and its putative role in signal transduction as a response to external stimuli. This type of response is crucial in pollen metabolism and development (including germination) and is mediated by both Ca^{2+} and specifically Ca^{2+}-binding proteins (Franklin-Tong, 1999). In many cases these proteins are cytosolic, while in others they are associated with membrane systems and form channels that generate characteristic gradients. The reported hydrophilic nature of Ole e 3 and the absence of specific anchoring sequences indicate that the allergen probably is not a protein intrinsic to the membrane. Its immunolocalization, on the other hand, relates this protein to cell endomembrane systems. In spite of the sparseness of information regarding its properties and probable functions, one hypothesis that fits both observations is that Ole e 3 may interact with other membrane proteins through a Ca^{2+}-dependent pathway. Ca^{2+}-sensor proteins like calmodulin behave in this manner (Valenta et al., 1998). Ole e 3 and in general the different polcalcins share many calmodulin structural features and were initially called "calmodulin-related proteins" (Ledesma et al., 1998). This further supports the hypothesis.

The hydrophilic nature of Ole e 3 would explain its location in both apertural and exine regions, even after fixation for microscopy using aqueous solutions. Localization of highly hydrophilic allergens in the walls, apertures or the "pollen coat" of pollen grains has often been achieved using anhydrous fixation (Grote, 1991) or cryotechniques (Alché et al., 1999), since they reduce protein elution into the aqueous fixative. Applying these types of technique would likely improve the localization of Ole e 3 allergen.

In situ amplification methods were used to detect Ole e 3 mRNAs, because the relatively low abundance of the latter falls below the threshold of standard in situ hybridization. The localization obtained here confirms the validity of this technique in spite of its procedural difficulty, particularly in TEM. The first development of this technique at plant ultrastructural level led to the localization of Ole e 1 transcripts in the pollen grain (Alché et al., 2002). In that case the transcripts were closely associated with the RER membrane system; the same cannot be clearly verified in this work for Ole e 3 mRNAs, which are more widely distributed throughout the cytoplasm than Ole e 1 mRNAs. The labeling observed on both nuclei is probably a consequence of the amplification of complementary genomic sequences. This signal is routinely eliminated in optical microscopy IS-PCR experiments by appropriate digestions with RNAase-free DNAase.

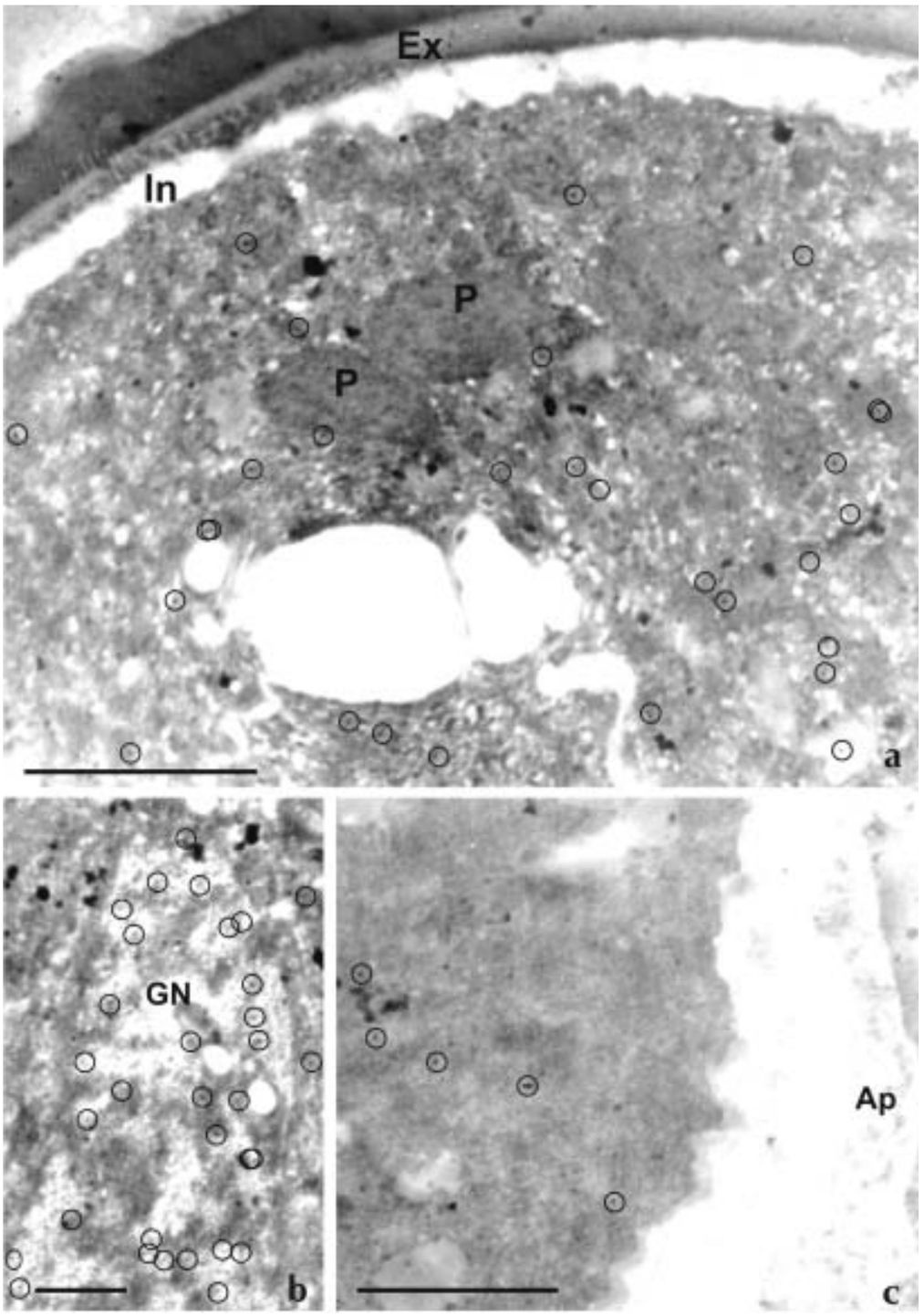

Fig. 4. Localization of Ole e 3 transcripts by TEM IS-RT-PCR. Gold particles are marked by circles. (**a**) Ole e 3 mRNAs are distributed throughout the cytoplasm and nuclei of both the vegetative and the generative cell. No gold particles are detected in vacuoles, lipid bodies, organelles or pollen wall, (**b**) Positive labeling associated to the chromatin in the generative cell nucleus, (**c**) Negative control performed by omitting the RT step. Ap – aperture; Ex – exine; GN – generative cell nucleus; In – intine; P – plastids. Bar in all figures = 1 μm.

However, digestion was not performed in this case due to the complexity of the TEM reaction.

The important role of Ca^{2+} in the pollen-stigma compatibility/incompatibility response and in pollen tube growth and orientation (Franklin-Tong, 1999) strongly suggests that the present study should be extended to both in vivo and in vitro germination of the olive pollen grain. One useful approach would be to combine simultaneous immunocytochemical localization of Ole e 3 with Ca^{2+} gradient detection methods based on microinjection of fluorochromes such as fura-2 dextran (Franklin-Tong, 1999).

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