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Localization of transcripts corresponding to the major allergen from olive pollen (*Ole e I*) by electron microscopic non-radioactive in situ RT-PCR

J.D. Alché, A.J. Castro, M.I. Rodríguez-García*

Departamento de Bioquímica, Biología Celular y Molecular de Plantas, Estación Experimental del Zaidín, CSIC, Profesor Albareda 1, 18008 Granada, Spain

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Abstract

In situ reverse transcription-PCR of mRNAs corresponding to the olive major allergen (*Ole e I*) has been tested at the ultrastructural level in mature olive pollen. The transcripts were present in the cytoplasm of both the vegetative and the generative cells, frequently associated to ribosomes in the endoplasmic reticulum. No labeling was detected in the pollen wall, nor in vacuoles, lipid bodies, plastids or mitochondria. Localization of the major olive allergen at ultrastructural level showed the protein present mainly in the lumen of the endoplasmic reticulum vesicles or pockets scattered in the cytoplasm, and in the outer region of the pollen exine. The results confirm the rough endoplasmic reticulum as the cell system involved in both the synthesis and storage of this protein. This is the first report of in situ RT-PCR on plant material at the ultrastructural level. The method described for mRNA amplification and detection is confirmed as a valuable tool for studying gene expression in plant material. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: Olive; *Olea europaea*; Pollen; Allergen; In situ RT-PCR; *Ole e I*

1. Introduction

Both the PCR (Polymerase Chain Reaction) and the in situ hybridization techniques (ISH) have separately proven their utility in the study of gene structure and function. In situ gene amplification methods combine the advantages of both techniques by increasing the number of specific target sequences that can then be readily detected by either in situ hybridization (indirect IS-PCR) or immunocytochemistry (direct IS-PCR). The combined use of both techniques allows the detection of target sequences under the threshold level for standard in situ hybridization, fixed in approximately 10–20 copies per cell for mRNA and 10 copies per cell for DNA (Komminoth and Long, 1993). The first report of this technique in single cells in suspension was that of Haase et al. (1990). Numerous protocols for IS-PCR have been developed by several groups since, and the number of potential research and diagnostic applications has grown in the last years. However, the methods used are not of general application and have to be adapted for each application. IS-PCR has been recently expanded to detect low copy number mRNAs after their reverse transcription (IS-RT-PCR), and has become a valuable tool for the study of gene expression.

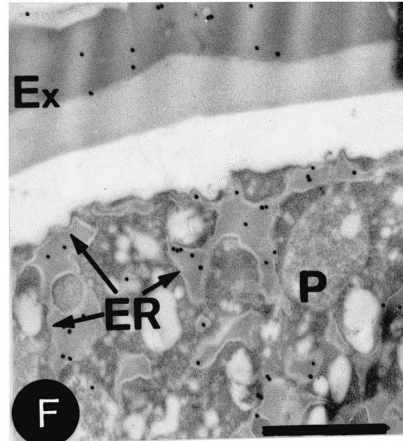
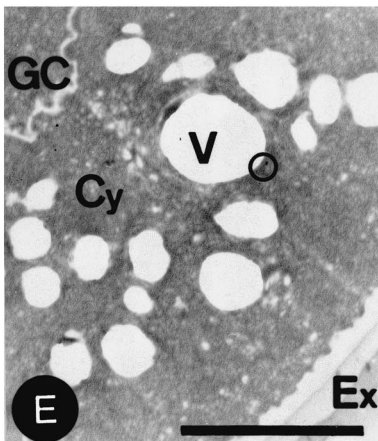
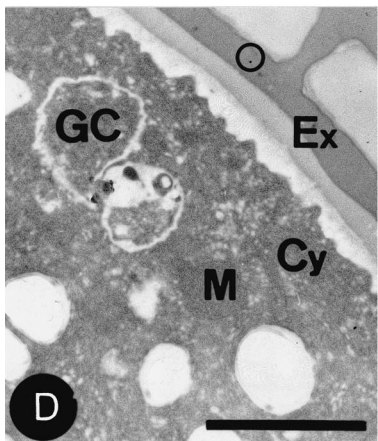
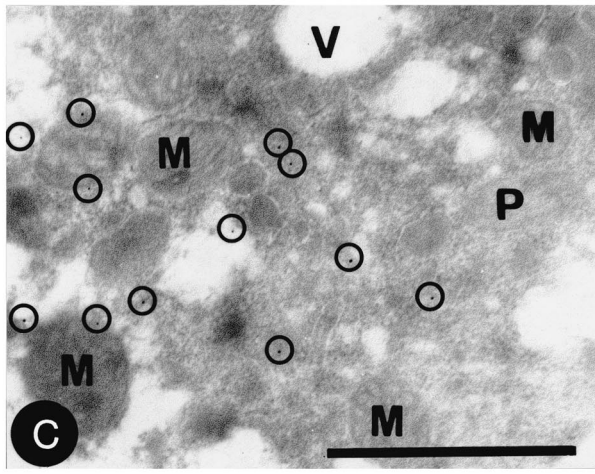
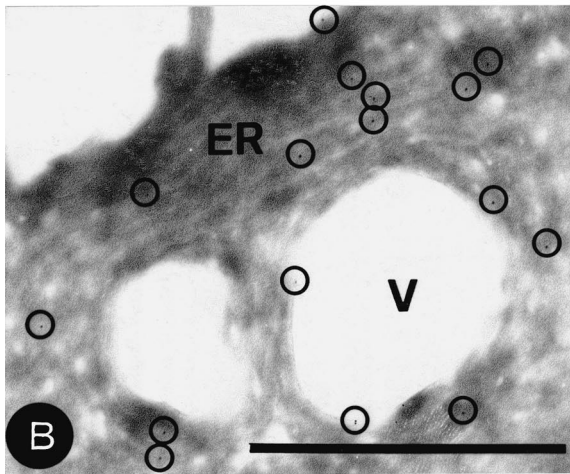
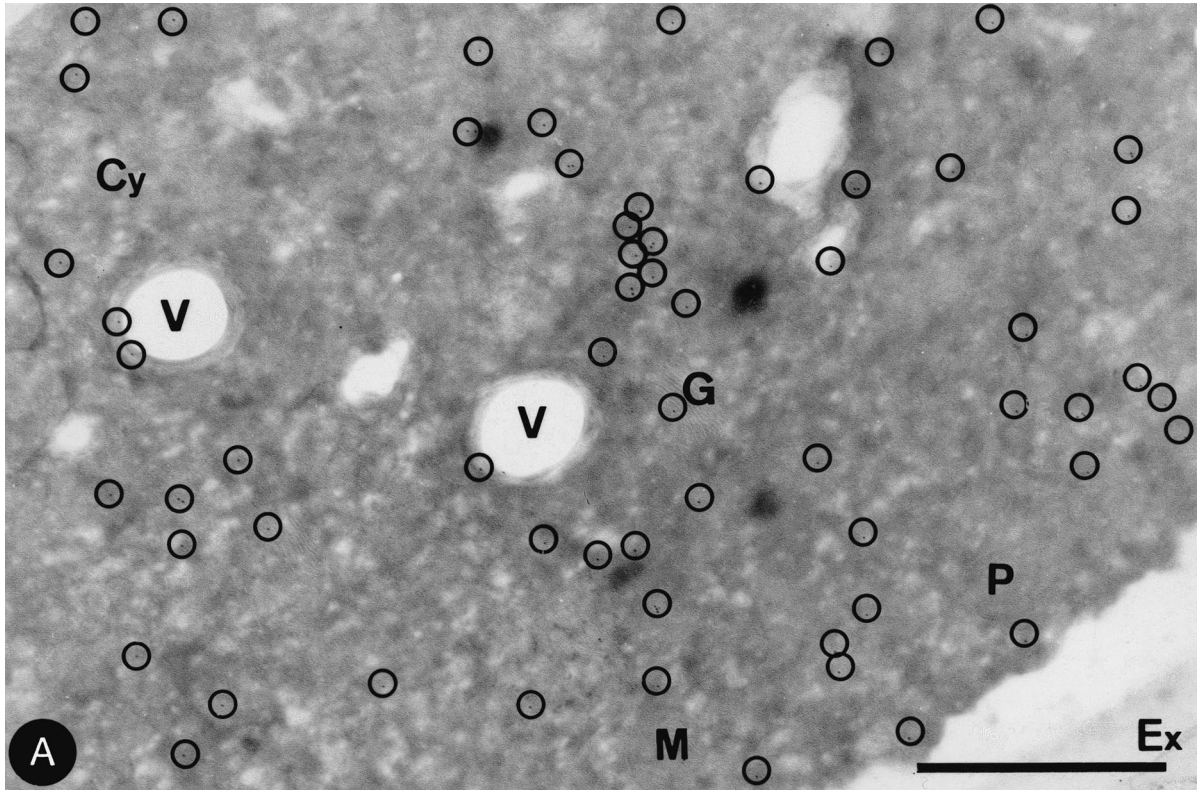
In spite of the overwhelming importance and potential of these techniques, two aspects are still underdeveloped: these techniques have rarely been used in plant studies and, on the other hand, their resolution is limited to the cellular level with only a few exceptions of studies made at the ultrastructural level. In this paper we describe for the first time the use of a IS-RT-PCR technique at the ultrastructural level in pollen grains of olive in order to detect the presence of transcripts corresponding to the major olive pollen allergen.

The olive (*Olea europaea* L.) is an important crop in Mediterranean countries and some areas of North America where its pollen represents one of the main causes of allergy by inhalation (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).

Transmission electron microscopy (TEM) immunocytochemical localization has been used to trace this protein throughout pollen development (Martín-Orozco et al., 1994; Rodríguez-García et al., 1995a, b). The allergenic protein is specifically located in the cisternae of the rough endoplasmic reticulum (RER) within the olive pollen grain.

* Corresponding author. Tel.: +34-958-121011; fax: +34-958-129600.

E-mail address: mirbicep@eez.csic.es (M.I. Rodríguez-García).



However, as is also the case for most pollen allergens, the biological function of these proteins remains unknown. In order to obtain clues regarding the biological role of the *Ole e I*, we have initiated a program in order to analyze the temporal and spatial expression patterns of *Ole e I* gene at different stages of anther development. This study involves a number of biochemical and molecular studies, as well as standard ISH (Alché et al., 1999). However, the use of TEM IS-RT-PCR techniques as shown here, will allow the detection of transcripts in the stages and tissues of this plant where those transcripts are relatively scarce and, therefore, undetectable by using standard ISH.

2. Materials and methods

2.1. Material preparation

Mature pollen was obtained from olive trees (*Olea europaea* L. cv. Picual) growing under natural conditions in several areas of the province of Granada (Spain) by vigorous shaking of dehiscent inflorescences placed inside large paper envelopes.

For RT-PCR in situ studies, the pollen was collected, purified by sieving through a 150 µm mesh, and immediately processed for electron microscopy according to Alché et al. (1998) with minor modifications. Briefly, the pollen was fixed in a mix of 4% paraformaldehyde, 0.1% glutaraldehyde in 0.1 M sodium cacodylate buffer pH 7.2 for 2 h at room temperature. The pollen was dehydrated in an ethanol series, gradually transferred to propylene oxide and embedded in Epon.

For *Ole e I* immunolocalization studies, the dehiscent pollen was subjected to cryofixation, freeze-substitution and low-temperature embedding in Unicryl resin (BBInternational, Cardiff, UK) as described in Alché et al. (1999). Ultrathin sections (80 nm) were obtained with a Reichert–Jung ultramicrotome and transferred to 300-mesh nickel grids.

2.2. IS-RT-PCR method

Pretreatment of the sections in order to promote access of reagents to the target sequences was carried out by floating the grids (section side down) onto a solution of proteinase K (Promega Corp. Madison, WI, USA) in 0.1 M Tris–HCl, 50 mM EDTA (pH 8.0). A wide range of concentrations and incubation times were assayed. Optimal treatment was

10 µg/ml for 15 min at 37°C. The reaction was stopped by addition of 0.1 M Glycine in phosphate buffered saline (PBS) for 5 min and further washing of the grids in PBS, (three times, 5 min each).

The grids were then placed into 0.5 ml PCR tubes, each one containing 65 µl of DEPC-treated H₂O and 5 µl of 540 ng/µl oligo-dT adaptor (5'-GACTCGAGTCGACATCGA(T)₁₇-3'). After incubation at 70°C for 10 min in a Progene thermocycler (Techne, Cambridge, UK), 2.5 µl of 10mM each dNTP, 5 µl of 0.1 M DTT, 20 µl of 5 × RT buffer and 2.5 µl of M-MLV reverse transcriptase (Promega Corp. Madison, WI, USA) were added. Reverse transcription was carried out after 10 min incubation at room temperature for 2 h at 42°C in the same thermocycler. The tubes containing the grids and the RT mix were heated to 70°C for 15 min, and finally left on ice for 1 min before proceeding to the next step.

The grids were transferred to new 0.5 ml-PCR tubes, each one containing 1 × PCR-buffer, dNTPs (1 mM dCTP, 1 mM dGTP, 1 mM dTTP, 0.75 mM dATP, 0.25 mM dig-dUTP Boehringer Mannheim GmbH, Germany), 25 ng of each primer (*Ole e I* and oligo dT-adaptor), 2.5 U DynaZyme DNA polymerase (Finnzymes Oy, Spoo, Finland) and water to 50 µl. In order to determine the optimal degree of amplification, the tubes were subjected to PCR amplification for 10, 15, 20, 25 and 30 cycles of 94°C for 1 min, 57°C for 2 min and 72°C for 1 min each. The oligonucleotide primer was designed based on the nucleotide sequence of *Ole e I* (Villalba et al. 1994): *Ole e I* primer (5'-ACCTCCAGTTTCTCAATTTTCAC-3'). A final step of amplification at 72°C for 5 min was carried out. After stringency washes (twice in 0.1 × SSC at 45°C for 20 min each), the grids were used for immunodetection.

A direct immunolabeling method was used to detect the PCR products: after blocking with 3% bovine serum albumin (BSA) in Tris buffered saline (TBS) for 1 h at room temperature, a goat anti-digoxigenin antibody conjugated to 10 nm gold particles (BBInternational, Cardiff, UK) diluted 1:25 to 1:100 in blocking buffer was applied to the grids for 1 h at room temperature. The grids were rinsed three times in TBS for 5 min, then in ultrapure water (three times, 5 min each), and finally stained with 5% uranyl acetate for 10 min. Observations were made in a Zeiss EM10C Transmission Electron Microscope operating at 60 kV. In several grids, a further silver enhancement treatment (silver enhancement kit, BBInternational, Cardiff, UK) was carried out for 5 min at room temperature in the dark before staining and observation.

Fig. 1. (A)–(E): Detection of *Ole e I* transcripts by IS-RT-PCR in ultrathin sections of mature pollen grains of olive. (A): Gold particles (circles) in the cytoplasm of the vegetative cell. The pollen wall, vacuoles, plastids and mitochondria appear unlabeled. (B): Detail of the rough endoplasmic reticulum showing intense labeling in the associated ribosomes. (C): Vegetative cell cytoplasm showing intense labeling. The organelles (plastids and mitochondria) are devoid of labeling. (D): Negative control prepared by omitting the reverse-transcription step. No labeling over the background is present (circle). (E): Negative control prepared by omitting both oligonucleotides from the PCR mix. Labeling is also practically absent. (F): immunolocalization of *Ole e I* protein in olive pollen grain: gold particles localize in the lumen of RER vesicles or pockets scattered in the cytoplasm, and in the outer pollen wall (exine). Cy: cytoplasm, ER: endoplasmic reticulum, Ex: exine, G: Golgi, GC: generative cell, M: mitochondria, P: plastid, V: vacuole. Scale bar indicates 1 µm.

Two negative controls for the RT-PCR reaction were used: a) some of the grids were not reverse transcribed, and b) *Ole e I* primer was omitted from the PCR mix for some of the grids. The remaining conditions were maintained for these controls.

2.3. Immunodetection of *Ole e I* allergen

Ultrathin sections were prepared as described for TEM in situ RT-PCR. Blocking of non-specific binding sites was carried out by incubation of the sections in 2% BSA, 1% normal goat serum in PBS (blocking solution) for 15 min. This was followed by treatment with anti-*Ole e I* monoclonal antibody diluted 1:10 in blocking solution for 1 h. The grids were washed in PBS and treated with goat anti-mouse IgG coupled to 20 nm gold particles (BBInternational, Cardiff, UK) diluted 1:50 in PBS for 1 h. After washing with PBS and ultrapure water, the sections were stained for 10 min in 5% uranyl acetate and observed.

3. Results

Gold labeling intensity and specificity were strongly dependent on many of the conditions used. However, the number of PCR cycles applied and the strength of both the proteinase K digestion and stringency wash were the most critical parameters for the reaction. Shorter treatments of proteinase K and/or lower concentrations than those recommended resulted in a poor signal after the whole procedure. This was due to low accessibility of the sample RNA to the following reactions. The opposite conditions (longer treatments and/or higher concentrations of proteinase K) resulted in a much stronger signal, though also in a clearly altered ultrastructure of pollen grains. This was a consequence of extensive enzyme activity. The conditions used in this work represent a compromise between maintaining a high sensitivity threshold, and a relatively unaltered ultrastructure. However, even slight differences in the thickness of the sections used may result in slight over/under treatments (compare ultrastructural details in Fig. 1(A)–(E)).

The optimal number of PCR amplification cycles also had to be determined empirically: reduced number of cycles resulted in low/no signal, whereas too many amplification cycles (over 20) were accompanied by a high background, as the consequence of the amplified sequences diffusing out from their original location in the thin section. Stringency washes helped to remove both unincorporated nucleotides and diffused products, also reducing nonspecific labeling.

Optimized IS-RT-PCR carried out in ultrathin sections of mature pollen grains showed gold particles decorating the cytoplasm of both the vegetative and the generative cells (Fig. 1(A)–(C)). Slight labeling also appeared in the nuclei of both cells corresponding to genomic sequences (not shown). Within the cytoplasm, the gold particles were frequently associated to ribosomes in the RER (Fig. 1(B)). No labeling was detected in the pollen wall, nor in vacuoles,

lipid bodies plastids or mitochondria (Fig. 1(C)). No significant gold labeling was found in negative control sections when the same treatment was applied, but the reverse-transcription step (Fig. 1(D)) or the oligonucleotides (Fig. 1(E)) were omitted.

Localization of the major olive allergen at ultrastructural level matched the results previously published by our group (Martín-Orozco et al., 1994; Rodríguez-García et al., 1995a,b): the major allergen mainly localizes in the lumen of the RER vesicles or pockets scattered in the cytoplasm (Fig. 1(F)). However, the use of low-temperature processing methods also allowed the ultrastructural localization of *Ole e I* in the pollen wall, whereas the nuclei, nucleolus, plastids and mitochondria were devoid of labeling.

4. Discussion

The first study of *Ole e I* expression in different tissues of olive tree was initiated by Villalba et al. (1994) using Northern blot analyses. These authors showed *Ole e I* transcripts to be present only in mature pollen and not in other tissues tested. Recently (Alché et al., 1999), we have investigated the distribution of *Ole e I* and its transcripts in the anther from premeiotic stages until the dehiscent pollen stage by using a variety of approaches including immunoblot detection, immunocytochemistry, RT-PCR analysis and standard ISH at both light microscopy and TEM level. Although we have drawn a picture of *Ole e I* expression during anther development, suggesting that this process is most likely controlled at the transcriptional level, a number of questions are still raised due to the difficulty of detecting low-abundant target sequences by ISH. Some of these questions include the ultrastructural localization of the mRNAs in the nutritious sporophytic tissue (tapetum), in the developing microspores at the early stages of transcript accumulation, and in the pollen grain and pollen tube immediately after hydration and during germination. For this purpose, the development of extremely sensitive techniques for the detection of target sequences is highly recommended. The application of IS-PCR at the ultrastructural level has to date been very limited (Cohen, 1996; Kareem et al., 1997). In plants, the use of IS-PCR techniques is completely unexplored, with a few exceptions: Johansen (1997) used IS-PCR and IS-RT-PCR to localize the gene coding for ribulose-1,5-biphosphate carboxylase/oxygenase and its transcripts with sub-cellular resolution, but at light microscopy. Deeken and Kaldenhoff (1997) used light microscopy IS-RT-PCR to detect the presence of transcripts for a light-repressible receptor protein kinase in cotyledons of etiolated seedlings of *A. thaliana*. We successfully show in this paper the localization by IS-RT-PCR of transcripts corresponding to *Ole e I* in the mature pollen stage. This is the first report of the use of this technique in plants at the ultrastructural level, and represents a substantial gain in sensitivity when compared to the localization of *Ole e I*

transcripts by using standard ISH techniques (Alché et al., 1999).

Immunocytochemical localization of *Ole e I* (Rodríguez-García et al., 1995a,b; Fernández et al., 1996) has documented the RER as the cellular system involved in the storage of allergenic proteins in several *Oleaceae* species. The present study, as well as the results of standard ISH techniques (Alché et al., 1999), confirm that the RER could be the preferential site of synthesis for these and other gametophytic proteins within the pollen grain, following the widespread pattern of synthesis in the RER-associated ribosomes and further export to the lumen of this membrane system (Larkins and Hurkman, 1978; Oparka and Harris, 1982; Krishnan et al., 1986). This therefore explains the high degree of differentiation reached by the RER during olive pollen maturation (Rodríguez-García and Fernández, 1990). This pattern has yet to be confirmed in the tapetum. However, a number of technical developments may further improve the quality of the localization. These include the use of alternative protocols for sample preparation (cryotechniques and low-temperature embedding), the standardization of the protease and amplification treatments, the inclusion of a DNAase step in order to remove genomic sequences, and the use of different sizes of gold-conjugates, not commercially available (or further silver enhancement techniques) for a better visualization of labeling.

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