

Cytochemical features common to nucleoli and cytoplasmic nucleoloids of *Olea europaea* meiocytes: detection of rRNA by in situ hybridization

J. D. Alché, M. C. Fernández and M. I. Rodríguez-García*

Plant Biochemistry, Molecular and Cellular Biology Department, Estación Experimental del Zaidín, CSIC, Profesor Albareda 1, E-18008 Granada, Spain

*Author for correspondence

SUMMARY

We used light and electron microscopic techniques to study the composition of cytoplasmic nucleoloids during meiotic division in *Olea europaea*. Nucleoloids were found in two clearly distinguishable morphological varieties: one similar in morphology to the nucleolus, and composed mainly of dense fibrillar component, and another surrounded by many ribosome-like particles. Cytochemical and immunocytochemical techniques showed similar reactivities in nucleoloids and the nucleolus: both are ribonucleoprotein in nature, and possess argyrophillic, argentaffinic and

highly phosphorylated proteins. Immunohistochemical techniques failed to detect DNA in either structure. In situ hybridization to a 18 S rRNA probe demonstrated the presence of ribosomal transcripts in both the nucleolus and nucleoloids. These similarities in morphology and composition may reflect similar functionalities.

Key words: nucleoloids, nucleolar proteins, rRNA, in situ hybridization

INTRODUCTION

Cytoplasmic bodies similar in morphology and ultrastructural characteristics to the nucleolus have been reported many times in relation to plant meiosis (Latter, 1926; Frankel, 1937; Hakansson and Levan, 1942; Gavaudan, 1948; Lindemann, 1956). These bodies have been given different names, although the term 'cytoplasmic nucleoloids' or simply 'nucleoloids', proposed by Dickinson and Heslop-Harrison (1970) for bodies found during microsporogenesis in *Lilium*, has been the most widely used.

Cytoplasmic bodies similar to the nucleolus have also been found in meristematic cells of different species (Sato and Sato, 1984; Sato et al., 1988). Whether these bodies are similar in function and physiological significance to the nucleoloids of microsporocytes is currently unknown.

The behavior and nature of nucleoloids have been studied in great detail in *Lilium* (Dickinson and Heslop-Harrison, 1970; Dickinson and Willson, 1985; Sato et al., 1989, 1991). These investigations have yielded information about the ribonucleoprotein nature of the nucleoloids, and the presence of rRNA transcripts. However, information concerning the presence of other components, and the behavior and function of nucleoloids compared with the nucleolus, is still scarce. It has been postulated that nucleoloids could be involved in the build-up of the post-meiotic ribosome population in the cytoplasm of the meiocyte (Dickinson and Heslop-Harrison, 1970). However it is important to note that nucleoloids have not been observed in all species, and thus cannot be considered a general feature of microsporogenesis. For example, no such bodies were found in *Beta vulgaris* (Majewska-Sawka, 1989) or *Lycopersicon escul-*

lentum (Carretero and Rodríguez-García, unpublished observations). The reason for this diversity is unknown.

Nucleoloids have rarely been studied in genera other than *Lilium*. Cytoplasmic nucleoloids are very common in *Olea europaea* during microsporogenesis and their large size and peculiar morphological characteristics make them a good material to study the behavior and nature of these structures (Rodríguez-García and Fernández, 1987). We report results obtained with several cytochemical and immunocytochemical techniques, and with in situ hybridization, used to search for components common to both cytoplasmic nucleoloids and nucleoli.

MATERIALS AND METHODS

Light microscopy and transmission electron microscopy

Short branches were cut from olive trees (*Olea europaea* L.) in different areas in southern Spain (Granada and Málaga provinces) during the flowering period.

After appropriate stages of the floral buds were roughly determined by their size, flowers were dissected to obtain anthers, which were fixed in 3% (v/v) glutaraldehyde and 4% (w/v) paraformaldehyde buffered in 0.1 M cacodylate (pH 6.8), for 90 minutes at room temperature (RT). The anthers were dehydrated in a series of ethanols, gradually transferred to propylene oxide and embedded in Epon.

Thin sections (1 µm) and silver defracting ultrathin sections were obtained with a Reichert-Jung ultramicrotome (Ultracut E), and transferred to glass slides or 400 mesh copper grids for light microscope (LM) or transmission electron microscope (TEM) observation, respectively.

For LM, the sections were stained with a mixture of Methylene Blue and Azure B (Humphrey and Pittman, 1974) for 20 minutes at

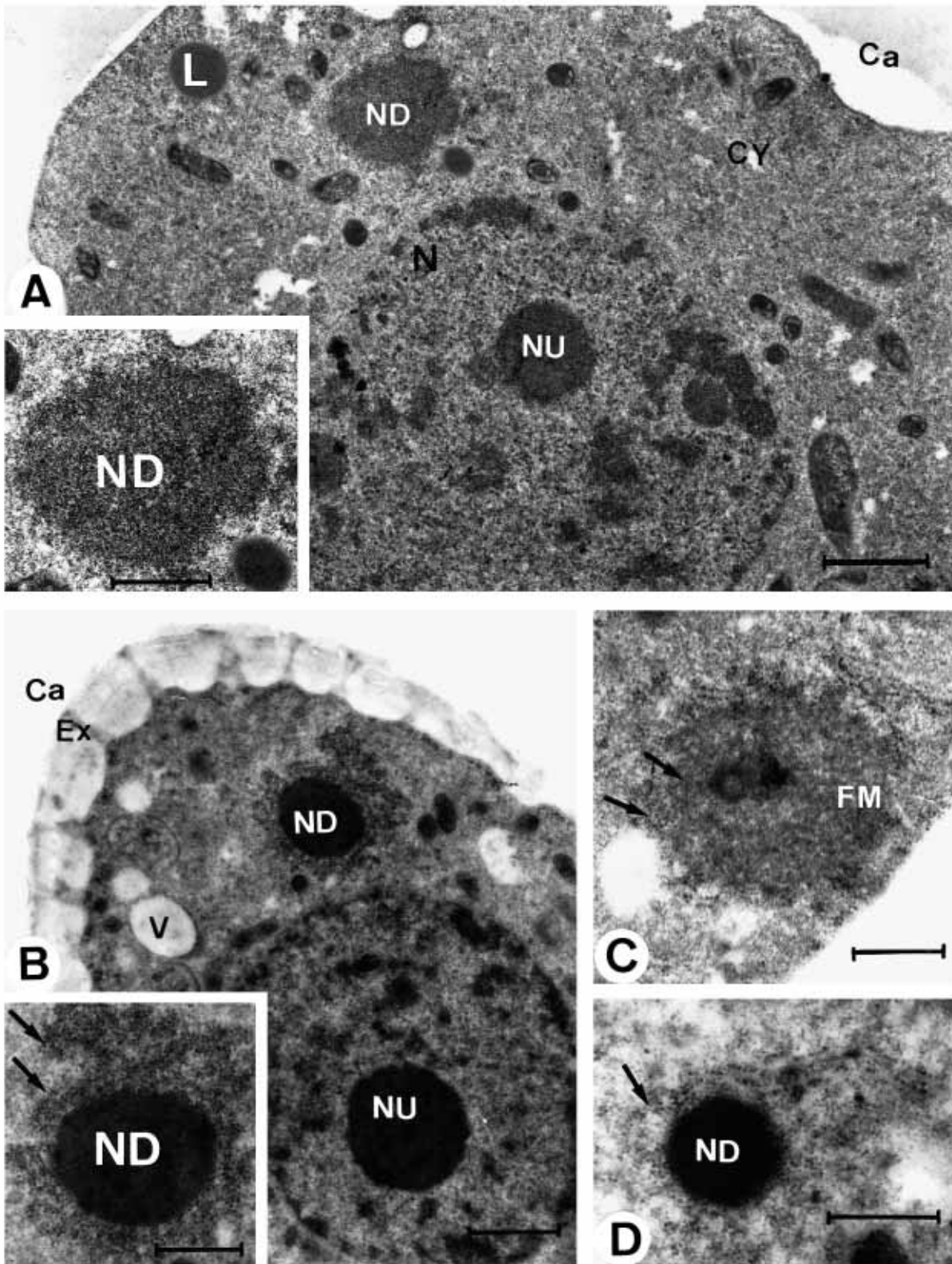


Fig. 1. Ultrastructural features of nucleoloids in *Olea europaea* microsporocytes. TEM. (A) Meicyte of *Olea europaea* at the young tetrad stage showing nucleoli (NU) and a cytoplasmic nucleoloid (ND) similar in shape and electron density to the nucleolus (inset). CA, callose; L, lipid body; N, nucleus; Cy, cytoplasm. Bar, 1 μ m (inset 0.5 μ m). (B) Meicyte at the young microspore stage. The nucleoloid (inset) is surrounded by ribosome-like particles (arrows). Ex, exine; V, vacuole. Bar, 1 μ m (inset 0.5 μ m). (C) Fibrillar mass (FM) in the cytoplasm of a young microspore in the tetrad. Ribosomes (arrows) are located at the periphery of this structure. Bar, 0.5 μ m. (D) Cytoplasmic nucleoloid in a young microspore surrounded by ribosome-like particles (arrows). Bar, 0.5 μ m.

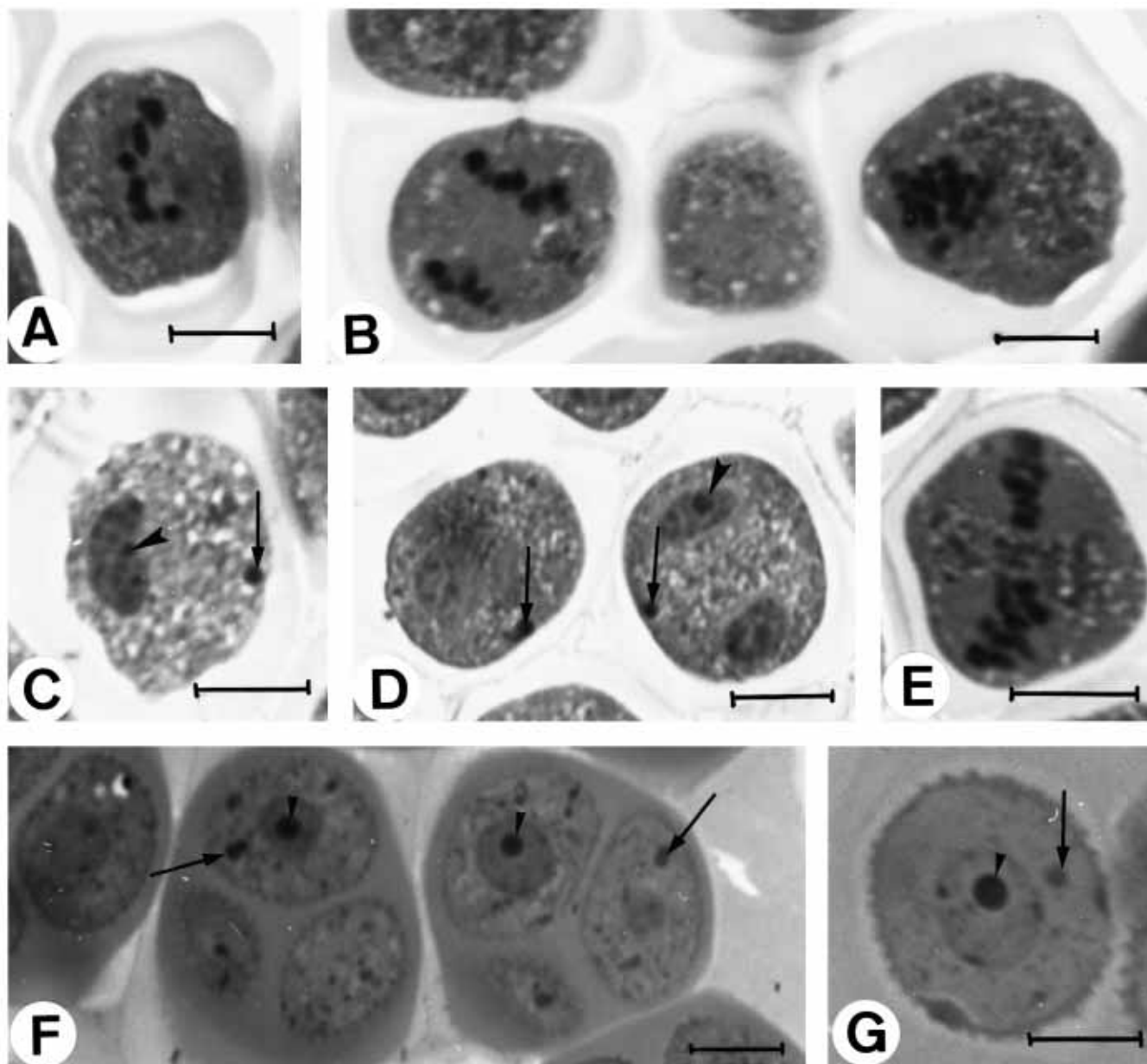


Fig. 2. Behaviour of nucleoloids during meiosis. *Olea europaea* meicytes after Methylene Blue-Azure B staining. LM. Bars, 5 μ m. (A) Meioocyte at metaphase I. No nucleoloid-like structures are stained in the cytoplasm. (B) Meioocytes at anaphase I (left) and early telophase I (right). No stained nucleoloids are visible in the cytoplasm. (C) Telophase I. A cytoplasmic nucleoloid stained in the cytoplasm (arrow). Nucleolus stained (arrowhead). (D) Cells at the dyad stage. Both the nucleolus (arrowhead) and cytoplasmic nucleoloids (arrows) are stained. (E) Meioocyte at the second meiotic division. The cytoplasm is apparently free of stained structures. (F) Cells at the tetrad stage. Nucleolus (arrowheads) and cytoplasmic nucleoloids (arrows) are again clearly stained. (G) Microspore shortly before release from the tetrad. Nucleoloids (arrow) are still visible as stained structures. The nucleolus (arrowhead) is also stained.

65°C. For TEM, ultrathin sections were contrasted with lead citrate and uranyl acetate, and observed in a Zeiss EM 10C TEM operating at 60 kV.

Acridine Orange stain

Glass slides with 1 μ m sections were treated with a freshly made saturated solution of sodium hydroxide in methanol for 2 hours to permeabilize the resin, then incubated for 15 minutes in a 0.1% (w/v) solution of Acridine Orange in PBS buffer in the dark. The slides were thoroughly washed in water, then mounted in citifluor-glycerol medium and observed in a Zeiss Axioplan microscope equipped with

an HBO 50 W epifluorescence system, using a combination of filters BP450-490, FT510, and LP520.

Argentaffinic reaction

Silver staining of argentaffinic proteins was performed in block after fixation using the method of Tandler and Pellegrino de Iraldi (1989), followed by processing as indicated for conventional LM.

Low temperature processing of samples

The anthers were fixed in 2% (w/v) paraformaldehyde, 0.1% (v/v) glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 1 hour at 4°C.

After fixation, samples were dehydrated in a series of methanols at progressively lower temperatures to -30°C , using an appropriately programmed freeze-substitution unit (FSU, Balzers Union).

Embedding in Lowicryl K4M resin and initial polymerization with ultraviolet light (2 days) were also done in the FSU at -30°C . Final polymerization of the Lowicryl blocks with UV light was done at RT for 2 days.

Silver staining

Pale gold sections of low temperature-processed samples were collected on 300 mesh gold grids. The grids were treated according to the method of Moreno et al. (1985) by floating them on 50 μl drops of the working solution (0.3% (w/v) formic acid, 0.6% (w/v) gelatin, 33% (w/v) silver nitrate) for 5 minutes in the dark. The grids were then washed in bidistilled water and floated on drops of 5% (w/v) sodium thiosulfate for 10 minutes, and observed after thorough washing without further contrasting.

Bismuth oxynitrate stain

Ultrathin sections of low temperature-processed samples were incubated with the working solution (1.3% (w/v) sodium tartrate, 0.6 M sodium hydroxide, 0.6% (w/v) bismuth oxynitrate in 0.1 M HCl-triethanolamine buffer, pH 7.4) for 1 hour, according to the procedure of Locke and Huie (1977), as modified by Puvion-Dutilleul and Laithier (1987). The sections were observed after thorough washing in bidistilled water without further contrasting.

DNA immunolabelling

Formvar-coated copper grids containing pale gold sections of low temperature-processed samples were treated essentially as described by Hansmann and Falk (1986): after unspecific blocking with PBSFT (5% (v/v) fetal calf serum, 0.1% (v/v) Tween-20 in PBS buffer), the grids were incubated in 1/5 anti-DNA (IgM) monoclonal antibody (Boehringer Mannheim) in PBSFT for 1 hour. As the second antibody, a polyclonal goat anti-mouse IgM conjugated to 10 nm gold particles (Biocell Laboratoires) was used, diluted 1/30 in PBSBT (1% (w/v) bovine serum albumin, 0.1% (v/v) Tween-20 in PBS buffer). Grids were incubated for 45 minutes in the second antibody and lightly stained with uranyl acetate before observation with TEM.

In situ hybridization

Preparation of samples

For in situ hybridization, anthers were processed at low temperature and embedded in Lowicryl K4M, or fixed and embedded in LR-Gold resin (London Resin Co.) according to the method of McFadden et al. (1988). In both cases, pale gold sections were collected on 300 mesh Formvar-coated gold or nickel grids.

Preparation of the probe

The single-stranded RNA probe was obtained after in vitro transcription of an *EcoRI/BamHI* fragment (1000 bp) of the 18 S rRNA gene of *Pisum sativum* (see Jorgensen et al., 1987), which was subcloned into pBluescript KS(+/-), kindly provided by G. I. McFadden (Plant Cell Biology Research Centre, University of Melbourne, Australia).

Transcription was begun at the T3 origin of the plasmid using bio-11-UTP nucleotide (Sigma) as the precursor; the method described by McFadden et al. (1989, 1990) was used to obtain a biotin-labelled antisense RNA, which served as the probe for in situ hybridization.

Transcription with the same labelled nucleotide, but beginning at the T7 origin, gave a biotin-labelled sense RNA, which was used as the control for in situ hybridization.

In both cases, the single-stranded biotinylated RNA was resuspended in hybridization buffer (50% (v/v) formamide, 0.75 M sodium chloride, 0.3 M trisodium citrate, 1 mM sodium pyrophosphate, 5 mM EDTA, 0.1% (w/v) Ficoll-400, 0.1% (w/v) PVP-40, 0.1% BSA, 1% (w/v) denatured herring sperm DNA in 50 mM phosphate buffer, pH 7.2).

Hybridization

Nucleic acids were made accessible on the ultrathin sections by incubating grids with 20 $\mu\text{g}/\text{ml}$ proteinase K (Boehringer Mannheim) in TE buffer (10 mM HCl-Tris, 1 mM EDTA, pH 7.5) for 2 hours at 37°C . Digestion was stopped by washing the grids repeatedly in TE.

For hybridization the grids were floated on 4 μl drops of the probe placed on a Petri dish cover inside a small hermetic hybridization chamber. This chamber, containing an excess of hybridization buffer, was incubated at 50°C overnight.

After hybridization, the grids were floated on 50 μl drops of 4 \times SSC (5 \times 1 minute at RT), 2 \times SSC (5 \times 1 minute at RT) and 1 \times SSC (2 hours at 50°C in a humid chamber) (1 \times SSC: 0.015 M sodium citrate, 0.15 M sodium chloride, pH 7.0).

The biotin-labelled hybrids were immunodetected by incubating the grids on 50 μl drops of SC buffer (0.5 M sodium chloride, 0.5% Tween-20, 50 mM PIPES, pH 7.2, for 15 minutes at RT) (McFadden et al., 1988), block buffer (1% BSA in SC buffer, 15 minutes at RT), goat anti-biotin polyclonal antibody (Vector Laboratories) diluted 1/20 in SC buffer (1 hour at 37°C in a humid chamber), SC buffer (5 \times 1 minute at RT), rabbit anti-goat IgG:10 nm gold (Biocell Research Laboratories) diluted 1/25 in SC (1 hour at 37°C in a humid chamber), SC buffer (5 \times 1 minute at RT), and bidistilled water (5 \times 1 minute at RT).

The grids were left to dry and then lightly stained with uranyl acetate before observation.

RESULTS

General characteristics of nucleoloids

The nucleoloids of *Olea europaea* are cytoplasmic bodies with no surrounding membrane, similar in shape and electron density to the nucleolus (Fig. 1). Nucleoloids ranged in size from 0.75 to 2.5 μm in diameter, with a mean of 1.40 μm . At the same stages, the nucleolus was larger, with a mean diameter of 2.7 μm . At high magnification, a fibrillar structure was discernible in the material making up the nucleoloids (Fig. 1A,D). This structure was clearly similar to that of the dense fibrillar component (DFC). During the tetrad stage in *Olea europaea* microsporocytes, the main component of the nucleoli was the DFC and the granular component (GC) was absent (Fig. 1A,B). Occasionally, electron-light areas similar to nucleolar vacuoles were noted inside the cytoplasmic nucleoloids.

Fig. 3. Cytochemical and immunocytochemical characterization of nucleoloids. (A) Meiocytes of *Olea europaea* at the tetrad stage. Acridine Orange stain. The nucleolus (arrowheads) and nucleoloids (arrows) appear as bright spots. Fluorescence microscopy. Bar, 10 μm . (B) Dyads of *Olea europaea* after the argentaffin reaction. Nucleoloids are intensely stained (arrows). LM. Bar, 10 μm . (C) Ag-NOR staining of a meiocyte in the young tetrad stage. The nucleolus (NU) and the nucleoloid (ND) are intensely stained. The precipitate is coarser toward the outside (arrows) and thinner toward the inside of the nucleoloid (inset). N, nucleus; CY, cytoplasm; Chr, chromatin. TEM. Bar, 0.5 μm (inset 0.25 μm). (D) DNA immunolabelling of a meiocyte at the young tetrad stage. The nucleoloid is completely devoid of gold particles. TEM. Bar, 1 μm . (E) Meiocyte in the tetrad. Bismuth oxynitrate staining. The nucleolus and nucleoloid show very dense deposits of bismuth on some areas of their surface (asterisks). Interchromatin and perichromatin granules are also stained (double arrows). TEM. Bar, 0.5 μm .

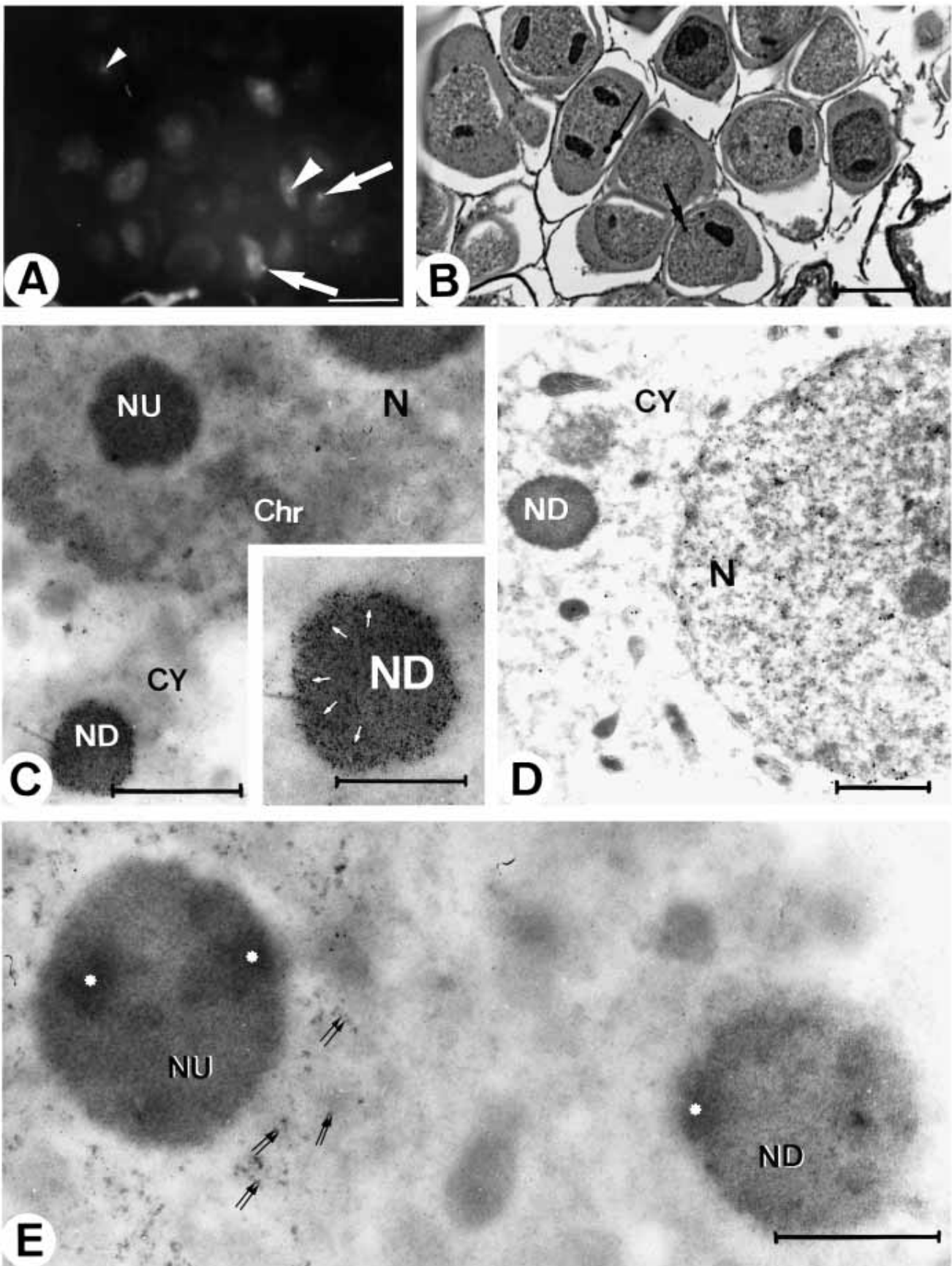


Fig. 3

Behavior of nucleoloids during meiosis

The presence of nucleoloids in the *Olea europaea* microspore was previously reported (Rodríguez-García and Fernández, 1987), although their complete development during microsporogenesis was not followed. In the present study, nucleoloids were found from telophase I until the free microspore stage in the anther locule (Fig. 2C,D,F,G). However, they were not observed during metaphase I, anaphase I (Fig. 2A,B), or during the second meiotic division (Fig. 2E). These bodies were numerous during the tetrad stage (Fig. 2F), and became less frequent as the ribosomal population increased during the free microspore stage.

During the late tetrad stage, when the first deposits of primexine appeared around the microspores, cytoplasmic nucleoloids were frequently surrounded by numerous ribosome-like particles or pre-ribosomal particles (Fig. 1B and inset). These particles were present around nucleoloids until the young microspore stage, when the cell is free in the anther locule.

Also during the late tetrad stage (shortly before the microspore is released from the callose wall), and throughout the young microspore stages, some irregularly shaped structures not surrounded by a membrane were seen in the cytoplasm. These bodies contained a fibrillar material of lower density than that in the nucleoloids (Fig. 1C), and were independent of the nucleoloids, although these were sometimes found nearby. These structures have already been described in more detail by our group as 'fibrillar masses' (Rodríguez-García and Fernández, 1987).

Cytochemical and immunocytochemical characterization

RNA detection

After staining of semithin sections with Azure B, which is specific for acid materials, both the nucleoli and nucleoloids were strongly stained blue under LM. No differences were seen between the two structures with regard to their level of staining (Fig. 2C,D,F,G).

At the fluorescence microscopic level, nucleoloids and nucleoli were similar in appearance after staining with the Acridine Orange method for nucleic acids. Both structures were brightly stained orange-red, whereas masses of condensed chromatin were weakly stained yellow (Fig. 3A).

Protein detection

In meiocytes, the argentaffin staining procedure clearly revealed nucleoli and cytoplasmic nucleoloids. Silver precipitate was also observed in the nucleoplasm, and a very light precipitate was seen in the cytoplasm (Fig. 3B).

After silver staining for argyrophillic proteins of the nucleolar organizer regions (Ag-NOR proteins), silver precipitate was observed in both the nucleolus and nucleoloids, whereas the nucleoplasm was nearly devoid of precipitate (Fig. 3C). This precipitate was distributed more or less homogeneously throughout the nucleolus, however, some small nucleolar vacuoles were devoid of precipitate. The nucleolus was composed mainly of DFC at this stage. Cytoplasmic nucleoloids exhibited a slightly different pattern, with two different densities of silver grains: staining of the inner area was similar to that in the nucleolus, whereas the silver grains in outer areas were large (Fig. 3C, inset).

Specific staining with bismuth oxynitrate appeared as extremely fine-grained deposits. The nucleoli were stained, with the exception of the small unstained nucleolar vacuoles. Staining was not homogenous, with alternating areas of greater and lesser density. Granules in the interchromatin and perichromatin region were strongly contrasted. In the cytoplasm the nucleoloids also show an uneven precipitate distribution similar to that of the nucleoli, with higher dense areas frequently occurring near the outer limit (Fig. 3E).

DNA detection

In assays based on the anti-double- and single-stranded DNA antibody, gold particles were most numerous in areas of high local DNA concentration, and in limited regions of organelles. However, gold particles were never found in or around the nucleoloids (Fig. 3D).

In situ hybridization to RNA

Hybridization of the probe in ultrathin sections led to widely distributed gold labelling in the cytoplasm, but not in the nucleoplasm, wall layers or callose wall (Fig. 4A). Both nucleoli and nucleoloids were intensely labelled (Fig. 4B,C,D). Gold particles were evenly scattered in the nucleolus, with the exception of small vacuoles that remained unlabelled (Fig. 4C).

Affinity of the cytoplasmic nucleoloids for the probe was identical to that seen for nucleoli (Fig. 4D). During the late tetrad stage, the ribosome-like particles present around the nucleoloids were also intensely labelled with gold particles. In this same stage, numerous labelled ribosomes were located along the periphery of the cell, close to the plasma membrane (Fig. 4B).

DISCUSSION

The results obtained with the different techniques used reveal a marked similarity between nucleoloids and the nucleolus, not only at the ultrastructural level, but also with respect to their most significant components as tested in this study. These similarities can be summarized essentially as the presence of rRNA and common proteins, especially those located in the DFC of the nucleolus. This DFC is the major component of the nucleolus during the tetrad and young microspore stages in *Olea europaea*, and seems also to be the main component of nucleoloids during these stages. The DFC has been considered

Fig. 4. In situ hybridization to 18 S rRNA probe. *Pisum sativum*. TEM. Antisense probe (A,B and C); control, sense probe (D). (A) General view of a meiocyte in the tetrad stage. In the nucleus gold particles are located mainly over the nucleolus; the cytoplasm is also labelled on the ribosome particles. CY, cytoplasm; N, nucleus; NU, nucleolus; P, plastid. Bar, 1 μ m. (B) Cytoplasm of a meiocyte in the late tetrad stage. The probe is bound to the nucleoloid (ND) and to the ribosomes in the cytoplasm, and is accumulated around the periphery of the cell (arrows). The ribosome-like particles around the nucleoloid are intensely labelled (arrowheads). Bar, 1 μ m. (C) Nucleolus of a meiocyte in the young tetrad stage showing affinity for the ribosomal probe. Nucleolar vacuoles appear devoid of gold particles. Bar, 0.5 μ m. (D) Nucleoloid and ribosome are practically free of gold particles. Bar, 0.5 μ m.

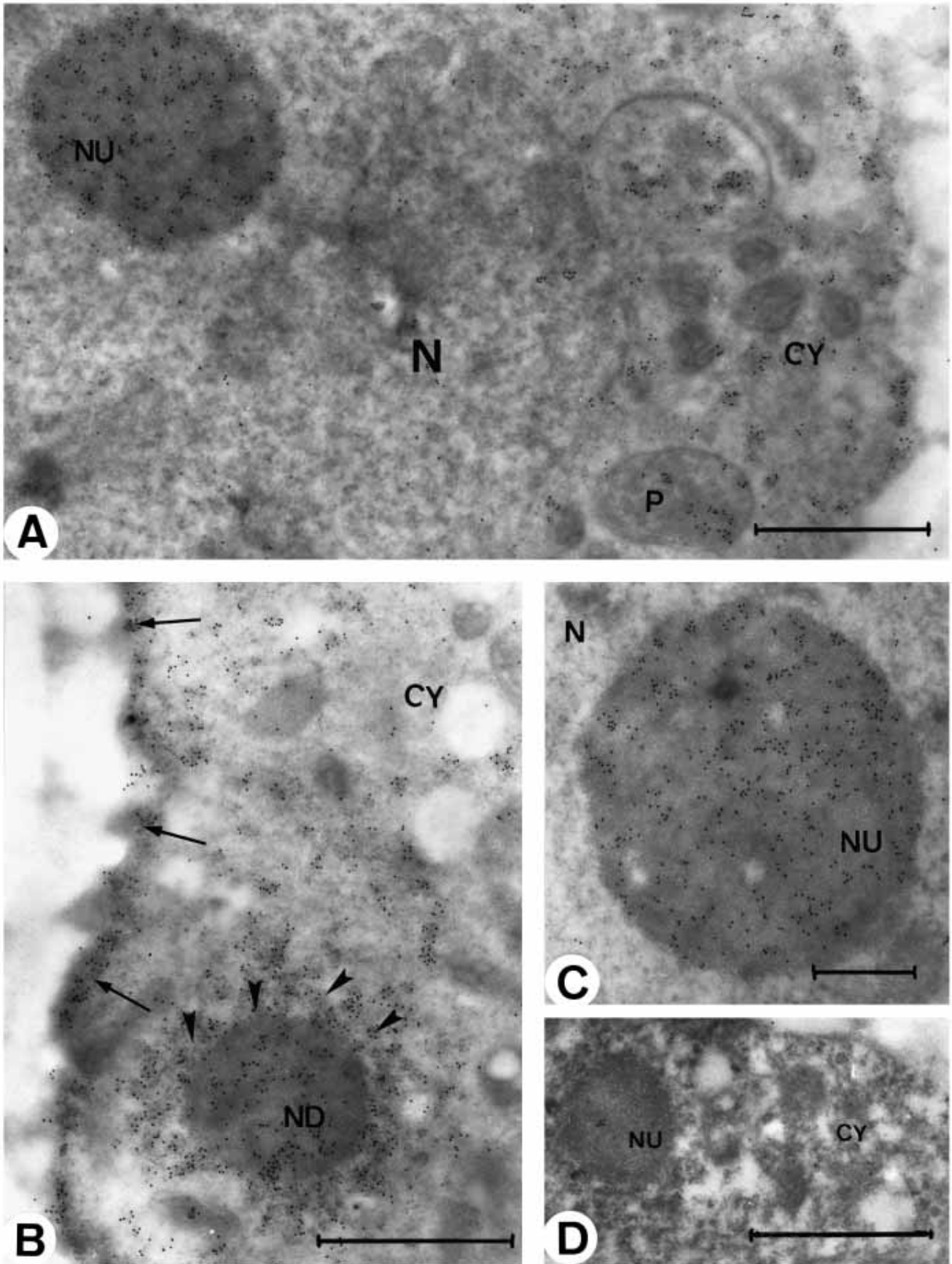


Fig. 4

traditionally as the site of transcription of ribosomal genes (reviewed by Goessens, 1984; Risueño and Medina, 1986). However, more recently there are conflicting data from different research groups showing either the presence of DNA and transcriptionally related proteins in the DFC and/or the fibrillar centres (see reviews by Deltour and Motte, 1990; Hernández-Verdun, 1991; Jordan, 1991; Raska et al., 1990; Scheer and Benavente, 1990; Swarczacher and Wachtler, 1991). These contradictory data make it difficult to resolve the question of where the transcription of the rRNA takes place. In any case, the apparent lack of DNA in the nucleoloids, together with the absence in these structures of regions analogous to the fibrillar centres, make transcription of ribosomal genes unlikely in the nucleoloids. In addition, the numerous ribosome-like particles around the nucleoloids suggest that these cytoplasmic structures are related to the assemblage and maturation of ribosomal subunits. The granular component of the nucleolus, considered the site of maturation and storage of ribosomal subunits (Goessens, 1984; Hernández-Verdun, 1986; Sommerville, 1986), cannot be distinguished as a component of nucleoloids. However, the presence of numerous ribosome-like particles around the nucleoloids during early microspore development suggests that ribosomes undergo a phase of maturation prior to their dispersal in the cytoplasm. These observations agree with the role attributed to nucleoloids by Dickinson and Heslop-Harrison (1970) in the restoration of the cytoplasmic ribosome population.

The positive staining of nucleoloids with Azure B and Acridine Orange was previously reported in *Lilium* (Dickinson and Heslop-Harrison, 1970) and *Maianthemum dilatatum* (Kusanagi and Kawano, 1975). The ribonucleoproteic nature of nucleoloids was also previously reported by the use of the EDTA technique in *Olea* (Rodríguez-García and Fernández, 1987) and *Lilium* (Sato et al., 1991). However, conclusive evidence of the presence of RNA is available only from techniques such as in situ hybridization, because of its specificity (McFadden, 1990). We took advantage of the highly conservative character of ribosomal genes in plants to localize sequences complementary to 18 S rRNA from *Pisum sativum* in the nucleolus and nucleoloids of *Olea europaea*, both of which showed similar patterns of labelling. An heterologous probe for ribosomal genes of wheat gave similar results in the nucleoloids of *Lilium* (Sato et al., 1991).

We have also demonstrated the presence of specific proteins in the nucleoloids, which reacted positively to bismuth oxynitrate, Ag-NOR and argentaffin staining. The two different silver staining methods used in this study are based on two different concepts such as 'argentaffinity' and 'argyrophilia', therefore they detect proteins of a different nature (Tandler, 1954). In both cases, the proteins responsible for positivity may be located in the same or in different structures (Risueño et al., 1990). The proteins responsible for the argentaffin reaction have not yet been identified, whereas some of the proteins responsible for silver staining have been well characterized as highly phosphorylated acidic proteins, containing thiol and disulfide groups (Sato and Bush, 1991). Two of the major silver staining proteins have been identified as nucleolar proteins B23 and C23 (Lichwe et al., 1979). The latter protein displays a strong similarity to the nucleolar 100 kDa, which has been proposed by Gas et al. (1984) as one of the proteins

also responsible for bismuth staining. The bismuth-staining procedure is highly specific for phosphorylated proteins (Locke and Huie, 1977).

Regarding the origin and formation of nucleoloids, it has been suggested that they or their precursors arise from the nucleolus-organizing region during pachytene and diplotene in the pollen mother cells and they are released into the cytoplasm during the meiotic divisions (Williams et al., 1973). The other possibility is that the nucleoloid material flows from the nucleus through the nuclear envelope. Nowadays the existence of 'shuttle proteins' (nucleolin and B23) is well known. They are involved in the intranuclear packaging of preribosomal particles, which shuttle constantly between nucleus and cytoplasm (Borer et al., 1989). Another shuttle protein, Nopp 140, was identified as a nuclear localization signal (NLS)-binding protein, which was shown to shuttle between the nucleolus and the cytoplasm (Meier and Blobel, 1992). This behaviour of these proteins might provide a reasonable explanation for the cytoplasmic occurrence of a DFC-related structure like the cytoplasmic nucleoloids. In order to clarify questions surrounding the formation and function of nucleoloids, it is important that future studies aim to determine the nature of nucleoloids and to compare them with specific nucleolar components.

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