Journal of Cell Science 112, 1065-1076 (1999)
Printed in Great Britain © The Company of Biologists Limited 1999
JCS9872

Ultrastructural distribution of a MAP kinase and transcripts in quiescent and cycling plant cells and pollen grains

G. Préstamo[1], P. S. Testillano[1,2], O. Vicente[3,*], P. González-Melendi[1], M. J. Coronado[1], C. Wilson[3], E. Heberle-Bors[3] and M. C. Risueño[1,‡]

[1]Centro de Investigaciones Biológicas, CSIC, Velázquez 144, E-28006 Madrid, Spain
[2]Centro de Biologia Molecular Severo Ochoa, CSIC/UAM, Cantoblanco, E-28049 Madrid
[3]Inst. Microbiology and Genetics, Vienna biocenter, Univ. Vienna, Dr Bohrgasse 9, A-1030 Vienna, Austria
*Present address: Inst. Biologia Molecular y Celular de Plantas, Univ. Politecnica de Valencia, Camino de Vera s/n, E-46022 Valencia, Spain
‡Author for correspondence (e-mail: cibr125@fresno.csic.es)

Accepted 18 January; published on WWW 10 March 1999

SUMMARY

Mitogen-activated protein kinases (MAPKs) are components of a kinase module that plays a central role in the transduction of diverse extracellular stimuli, including mitogens, specific differentiation and developmental signals and stress treatments. This shows that reversible protein phosphorylation cascades play a pivotal role in signal transduction in animal cells and yeast, particularly the entry into mitosis of arrested cells. Homologues of MAPKs have been found and cloned in various plant species, but there have been no data about their in situ localization at the subcellular level and their expression in plant cells so far. In the present paper we report the first data on the ultrastructural in situ localization of MAPK and their mRNAs in various plant cells.

Proliferating and quiescent meristematic plant cells were studied to evaluate whether changes in MAPK presence, distribution and expression accompany the entry into proliferation of dormant cells. Moreover, MAPK localization was analyzed in vacuolate microspores. Polyclonal antibodies against the deduced MAPK from the tobacco Ntf6 clone were able to recognize homologue epitopes by immunocytochemical techniques in the cell types studied. The pattern of protein distribution is similar in all the cases studied: it is localized in the cytoplasm and in the nucleus, mainly in the interchromatin region. The quantitative study of the density showed that MAPK labelling is more abundant in cycling than in quiescent cells, also suggesting that, in plants, MAPK pathways might play a role in cell proliferation. RNA probes for conserved regions of the catalytic domain of plant MAPK homologue genes were used to study MAPK expression in those plant cells. In situ hybridization (ISH) showed the presence of MAPK transcripts in the three plant cell types studied, but levels were very low in quiescent cells compared to those in cycling cells. The quantification of labelling density of ISH signals strongly suggests a higher level of MAPK expression in proliferating cells, but also some basal messenger presence and/or expression in the quiescent ones. Immunogold and ISH results show the presence and distribution of MAPK proteins and mRNAs in vacuolate microspores. This represents a very dynamic stage during pollen development in which the cell nucleus is being prepared for an asymmetrical mitotic division, giving rise to both the generative and the vegetative nuclei of the bicellular pollen grain. Taken together, the data indicate a role played by MAPK in the re-entry into proliferation in plant cells.

Key words: MAP kinase, Plant cell, Cell proliferation, Pollen grain, Immunoelectron microscopy, In situ hybridization

INTRODUCTION

MAPKs constitute a family of serine/threonine protein kinase that has been highly conserved during evolution, with members found in such diverse organisms as mammals, *Xenopus* and yeast (Pelech and Shanghera, 1992; Blumer and Johnson, 1994; Davis, 1994; Marshall, 1994; Herskowitz, 1995; Schultz et al., 1995). Their activation requires phosphorylation on both threonine and tyrosine residues (Posada and Cooper, 1992; Payne et al., 1991). Progress in understanding signal transduction in animals and yeast has shown that reversible protein phosphorylation plays a pivotal role in many signalling cascades, including the entry into mitosis of arrested cells (Meloche et al., 1992; Chen et al., 1992; Ruderman, 1993).

In spite of the extensive knowledge about molecular and biochemical characterization of MAPK in vertebrates and yeast, much less information is known about plant MAPKs and their functions. The presence of MAPK homologues in plants has been demonstrated in several plant species (Wilson et al., 1993, 1995; Duerr et al., 1993; Jonak et al., 1993, 1994, 1995; Mizoguchi et al., 1993, 1994; Stafstrom et al., 1993; Decroocq-Ferrant et al., 1995). The corresponding clones and deduced

proteins in plants share various molecular characteristics, typical of the MAPK family of other eukaryotes. Although our present knowledge on the possible biological roles of plant MAP kinases is lagging far behind that of their yeast and animal counterparts, there is now evidence that, also in plants, MAP kinases are also involved in signal transduction pathways which mediate cellular responses to a variety of external stimuli, including hormones, fungal elicitors, wounding or mechanical manipulation, and other abiotic stress conditions such as drought, cold or high salt (see Hirt 1997, for a recent review). Recently, evidence for the involvement of a plant MAPK in mitosis has been reported (Calderini et al., 1998). In several cases, distinct MAP kinases have been identified within the same species, which show relatively low homology (50-70% identity at the amino acid level) and probably fulfil different biological functions, being specifically involved in the response to different stimuli. On the other hand, much higher identities have been found between specific MAP kinases from different species. For example, the tobacco MAP kinases Ntf3, Ntf4 and Ntf6 (Wilson et al., 1995) show identity scores in the 51-67% range for the different pairs; however, Ntf4 is 92% identical to the alfalfa MAP kinase MsK7 (MsERK1) (Jonak et al., 1993, 1995; Duerr et al., 1993) and 90% identical to the D5 protein from pea (Stafstrom et al., 1993), while tobacco Ntf3 (Wilson et al., 1993) is similar (83-84% identity) to ATMPK1 and ATMPK2 from *Arabidopsis* (Mizoguchi et al., 1994). It seems logical to assume that these two groups of proteins are functional homologues, derived from distinct ancestral MAP kinase genes already present before the divergence of these species.

Most of these reports on plant MAPK have been performed at the molecular level but there is no available data about in situ localization at the subcellular level of these proteins in plant cells. Previously, a few papers on mammalian cells have reported in situ localization of MAPK, but only at the light microscopy level (Shangera et al., 1992; Chen et al., 1992), and only a recent publication reports data on the localization of a plant MAPK in tobacco cells by immunofluorescence experiments (Calderini et al., 1998). Scarce information is available about MAPK expression in plants (Jonak et al., 1993, 1994, 1995, 1996; Decroocq-Ferrant et al., 1995) and no in situ hybridization experiments localizing MAPK mRNAs have been reported until now.

In the present paper, the first data (since our preliminary report; Préstamo et al., 1997) on ultrastructural in situ localization of MAPK proteins and their transcripts in various types of plant cells are reported. Proliferating and quiescent meristematic plant cells are studied to evaluate whether changes in MAPK presence, distribution and expression accompany the entry into proliferation of dormant cells. Moreover, MAPK localization is analyzed in vacuolate microspores, which divide to produce pollen grains that contain two unequal cells, the vegetative and the generative. The stage of vacuolate microspore is also known to be able to respond to stress conditions and deviate towards haploid embryogenesis (González-Melendi et al., 1995, 1996a,b; Testillano et al., 1995a).

Polyclonal antibodies against the deduced MAPK from the tobacco Ntf6 clone (Wilson et al., 1995) are able to recognize homologue epitopes in the plant cells studied. In situ hybridization assays with conserved regions of the catalytic domain of plant MAPK homologue genes also indicate MAPK expression in those plant cells.

The results show for the first time the ultrastructural localization of MAPK proteins and transcripts in plant cells at different states of metabolism/activity (quiescence, proliferation, in development), revealing differences among them. This data will shed light on the involvement of this family of proteins on various plant cell processes such as proliferation and development.

MATERIALS AND METHODS

Plant material

The material used was proliferating and quiescent *Allium cepa* L. (onion) root meristems from germinating and non-germinating bulbs, respectively, and *Capsicum annuum* L. (pepper) anthers containing pollen grains at the developmental stage of vacuolate microspores. Quiescent roots were directly excised from non-germinating onion bulbs. Proliferating meristems were obtained from onion bulbs germinated in water at 15°C under standard conditions. Pepper anthers were carefully excised from flower buds of plants growing in a greenhouse under controlled temperature (23°C) and photoperiod (14 hours light). Some samples were used for immunofluorescence assays as described in the corresponding section. Other samples were processed in the same way for immunoelectron microscopy and in situ hybridization.

Probes and antibodies

PCR with two degenerate oligonucleotide primers was used to amplify DNA fragments, corresponding to part of the catalytic domain of MAP kinases, from a tobacco cell suspension cDNA library. The forward primer, 5′-CGCGAATTCGGN(G/A)A(G/A)GGN-GCNTATGG, was based on the conserved ATP binding site of MAP kinases in subdomain I (GXGAYG) and contained the underlined *Eco*RI site. The reverse primer, complementary to the phosphorylation site in subdomain VIII (TEYVVT), had the sequence 5′-CTCGGATCCNGTNACNAC(G/A)TA(C/T)TCNGT, and included a *Bam*HI site (underlined). After 40 cycles of PCR, the expected amplified fragments of ca. 450 bp were isolated from an agarose gel, digested with *Eco*RI and *Bam*HI and cloned into pBluescript SK– (Stratagene) previously digested with the same enzymes. One of the isolated plasmids, containing the tobacco MAP kinase Ntf5 PCR fragment (Wilson et al., 1995), was linearized with *Eco*RI and used to synthesize a DIG (digoxigenin)-labelled anti-sense RNA probe by in vitro transcription with T3 RNA polymerase and the DIG-RNA labelling kit (Boehringer Mannheim), as recommended by the manufacturer. The corresponding sense RNA probe was similarly obtained by transcription from the T7 promoter, after linearization with *Bam*HI. Polyclonal antibodies were produced in rabbits immunized with a synthetic peptide (NEALKFDPNTMK), corresponding to the last 12 amino acids of the tobacco MAP kinase Ntf6 (Wilson et al., 1995), and purified by binding to Protein A-Sepharose beads.

Immunoblotting

Immunoblot experiments were performed with total protein extracts of the plant samples and the anti-Ntf6 antibody. The procedure was that previously described in detail by Testillano et al. (1993). Briefly, total protein extracts of tissue homogenates was obtained using a cracking buffer containing 2% sodium dodecyl sulfate (SDS) and 1% β-mercaptoethanol. Proteins were separated by electrophoresis in 12% acrylamide-SDS gels and transferred to Immobilon membranes (Millipore Corporation, Bedford MA/USA). Prestained standard proteins (Bio-Rad) were also separated in the same gel and also

transferred to the membranes. The sheet from a single gel was cut and used either to stain the total proteins transferred, with 0.1% Amido Black, or for incubation with the antibody. For the immunodetection, the strips were blocked in 2% powdered skimmed milk, 0.05% Tween-20 in Tris-buffered Saline (TBS), at 4°C overnight. Then, they were incubated for 2 hours with the anti-Ntf6 (P6) antibody diluted 1:3000 in the blocking buffer. After three rinses in the same buffer, they were incubated for 1 hour with alkaline phosphatase-conjugated anti-rabbit IgG (Boehringer Mannheim), diluted 1:1000 in the same buffer. Finally, the proteins recognized by the antibody were revealed by treatment with a nitro blue tetrazolium, bromo-chloroindolyl-phosphate (NBT-BCIP) mixture. Control incubations were done by replacing the first antibody with the buffer.

Immunofluorescence and confocal microscopy

Bulbs of *Allium cepa* L. were germinated in standard conditions. After two days, the very tips of the roots were carefully excised and fixed in 4% (w/v) formaldehyde in PBS for 2 hours at room temperature. Then, the samples were washed for 15 minutes in PBS, three times. 30 μm vibratome (Bio-Rad) sections were obtained and placed onto glutaraldehyde activated and 3-aminopropyltrietoxysilane coated slides. Sections were permeabilized with 2% (w/v) cellulase (Onozuka R-10) in TBS (25 mM Tris-HCl, pH 7.4, 140 mM NaCl, 3 mM KCl) for 30 minutes at room temperature and used immediately for immunofluorescence assays. After three washes in TBS, the sections were incubated with 5% BSA in TBS for 5 minutes and then with the first antibody (anti-MAPK) diluted 1/50 in TBS for 1 hour at room temperature. After three rinsing steps in TBS for 15 minutes each, the secondary antibody (anti-rabbit-TRITC) diluted 1/200 in TBS, was applied for 45 minutes at room temperature. Finally the sections were washed in TBS and stained with DAPI. Confocal optical sections were collected using a Bio-Rad MRC-1000 confocal scanning head mounted on a Zeiss Axiovert 135 microscope. Pictures were taken from the projection of a series of 10 to 15 optical sections.

Processing for electron microscopy

Samples were fixed in 4% paraformaldehyde in PBS, pH 7.3, at 4°C, overnight. After several rinsings in PBS, they were dehydrated in a methanol series at 4°C and subjected to the methylation-acetylation (MA) treatment (Testillano et al., 1995b) to improve the visualization of the ultrastructure. Then, samples were washed in pure methanol, infiltrated and embedded in Lowicryl K4M at −30°C and polymerized under ultraviolet irradiation.

Lowicryl ultrathin sections were placed on colodion and carbon-coated nickel grids and used for immunogold labelling and in situ hybridization. Some sections were directly contrasted with uranyl acetate and lead citrate to study the ultrastructural organization of each plant cell type.

Immunogold labelling

Immunogold labelling was performed essentially as previously described by us (Risueño and Testillano, 1994; Mena et al., 1994). Grids carrying Lowicryl ultrathin sections from MA-treated samples were floated for some minutes on drops of double-distilled water, PBS and 5% bovine serum albumin (BSA) in PBS. Then, they were incubated with anti-MAPK polyclonal antibody (P6) diluted 1/25 in 1% BSA in PBS, for 1 hour at room temperature. After three washes in 1% BSA, sections were incubated with a goat anti-rabbit IgG conjugated to 10 nm colloidal gold (BioCell, Cardiff, UK) diluted 1/25 in 1% BSA in PBS, for 45 minutes. Then, the grids were washed in PBS and double-distilled water and air dried. Finally, sections were counterstained with 2% aqueous uranyl acetate for 10 minutes, and lead citrate for 15 seconds, and observed in a JEOL 1010 electron microscope at 80 kV.

Controls were performed avoiding the anti-MAPK antibody during the first incubation.

Ultrastructural cytochemistry for ribonucleoproteins

EDTA regressive staining, preferential for ribonucleoproteins was performed on grids after the immunogold labelling (Testillano et al., 1993), to identify the nuclear subcompartments in which the labelling appeared. Lowicryl ultrathin sections were subjected to immunogold labelling, washed and air dried, then stained with 2% uranyl acetate for 20 minutes, EDTA solution for 10 seconds and lead citrate for 20 seconds.

In situ hybridization

RNA/RNA ultrastructural in situ hybridization was performed on Lowicryl ultrathin sections from MA-treated samples using digoxigenin-labelled RNA probes. Lowicryl ultrathin sections were pretreated with 1 μg/ml Proteinase K in 0.1 M Tris-HCl, pH 7.5, containing 50 mM EDTA, for 1 hour at room temperature. After three 10 minute washes with the same buffer, and one rinsing in double-distilled water, they were air dried.

For the hybridization, each grid carrying sections was incubated with 25 μl of hybridization solution, at 50°C, overnight. To avoid evaporation of the solution, drops of hybridization solution were put on multiwheel slides (ICN Pharmaceuticals Inc. Costa Mesa, CA, USA) and placed in a moist chamber. The hybridization solution consisted of digoxigenin-labelled RNA probe diluted 1/25 in the hybridization buffer (50% formamide, 10% dextran sulfate, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 300 mM NaCl, 200 μg/ml yeast tRNA). Then, grids were washed 4 times, 2 minutes each in 4× SSC (1× SSC consisting of 150 mM sodium chloride, 15 mM sodium citrate, pH 7.2) and 2× SSC four times, 2 minutes each at room temperature. Sections were finally washed at 50°C in 1× SSC, two times, 1 hour each. The visualization of hybrids was performed by immunogold labelling with anti-digoxigenin-gold antibodies. After the washes in SSC, the grids were floated for some minutes on PBS and 5% BSA in PBS. Then, they were incubated with goat anti-digoxigenin conjugated with 10 nm colloidal gold (BioCell, Cardiff, UK) diluted 1/25 in 1% BSA for 45 minutes. Finally, the sections were washed in PBS and double-distilled water, air dried and counterstained with uranyl acetate and lead citrate as described for immunogold experiments. They were observed in a JEOL 1010 EM at 80 kV.

Controls were performed replacing the antisense RNA probe by the sense probe at the same concentration in the hybridization solution.

Quantitative evaluation

To evaluate labelling density in proliferating and quiescent cells in various cellular subcompartments, 15-25 micrographs were analyzed after immunocytochemistry and in situ hybridization experiments in each cell type. The micrographs were obtained from separated cells and taken from three different grids in each case. The cellular subcompartments considered for quantification were cytosol, cytoplasmic vacuoles, condensed chromatin masses and the interchromatin region.

The outlines of the different cell components selected for analysis were randomly selected and drawn, and the area of each one was estimated in μm^2 by point-counting procedures. The number of individual gold particles localized in each selected area was hand-counted and labelling density was measured as the number of gold particles/μm^2. The minimum sample size for each parameter and cell component considered was determined by the progressive mean technique (confidence limit 5%). The density labelling over the different cellular subcompartments was finally estimated using a BASIC program developed in Dr J. Renau-Piqueras' laboratory (La Fe Hospital, Valencia, Spain).

The density labelling on cytoplasmic vacuoles was taken as the inner control of the background level. Results of the quantitative analysis were summarized in histograms.

RESULTS

Immunoblotting with anti-MAPK antibodies

Immunoblotting of the anti-Ntf6 (P6) polyclonal antibody from tobacco (Wilson et al., 1995) on onion root meristem protein extracts revealed one single band of approximately 43-45 kDa molecular mass (Fig. 1). A weak and diffuse background appeared on several regions of the strips. Controls avoiding the first antibody did not show any signals.

This result suggests that the anti-Ntf6 antibody recognizes a MAPK that has been conserved between onion and tobacco. The immunoblot experiments illustrate the specificity of the in situ signal provided by the antibody on the plant cells studied.

Immunolocalization of MAPK

Polyclonal anti-MAPK antibodies were used for immunofluorescence and immunogold assays on different plant cell types.

Cycling and quiescent onion root meristems

Immunofluorescence has shown a positive signal in onion root meristems, the signal being more intense on proliferating than on quiescent cells. The cytoplasm showed positive immunofluorescence, as an heterogeneous signal, in both cell types, the nuclear border displaying greater brightness in some regions and cells (Fig. 2). In proliferating cells, some nuclei exhibited a weak non-uniformly distributed fluorescence signal (Fig. 2B).

For immunogold labelling experiments, samples were cryoprocessed and embedded in Lowicryl. The methylation-acetylation (MA) cytochemical method was applied to improve the visualization of the nuclear structures (Figs 3, 5 and 6).

In quiescent cells of onion root meristems, the nucleus shows compact chromatin masses forming a large reticule with roundish limits (Fig. 3A). The interchromatin region occupies large nuclear areas and displays numerous granular structures and nuclear bodies of various types. The nucleolus exhibits a structural organization typical of an inactive state, which is compact and mainly formed by dense fibrillar component (Fig. 3A). In the cytoplasm, small vacuoles and various organelles are present in ribosome rich areas (Fig. 3A).

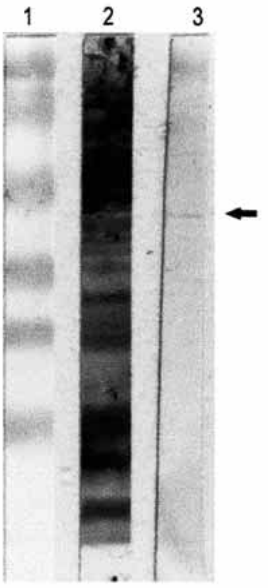

Fig. 1. Immunoblotting of onion root meristem total protein extracts with anti-MAPK antibodies. Lane 1, molecular mass standards representing from top to bottom: 103.0, 77.0, 48.0, 34.2, 28.4 and 20.5 kDa. Lane 2, total proteins transferred, Amido Black staining. Lane 3, incubation with anti-MAPK antibody, it recognizes one band of approximately 43-46 kDa (arrow).

When anti-MAPK antibodies were used for immunogold assays on quiescent cells, labelling was mainly distributed over the cytoplasm and the nucleus, with the rest of the cellular compartments, like cytoplasmic vacuoles and cell walls, showing only very few gold particles, corresponding to unspecific background binding (Fig. 3C). Occasionally, labelling was observed on the endoplasmic reticulum and/or some cytoplasmic vesicles. In the nucleus, gold particles mainly appeared dispersed on the interchromatin region (Fig. 3C); some scarce labelling was also observed on condensed chromatin masses.

The ultrastructural organization of cycling cell nuclei from onion root meristems depends on the cell cycle period. In this study only interphasic cells were analyzed. They exhibited large nuclei with condensed chromatin masses of different sizes and degrees of condensation, and showed abundant fibres at their periphery. The interchromatin region was very rich in fibrillo-granular structures (Fig. 3B). The nucleolus was large and shows the granular component intermingled with the dense fibrillar one. In the latter, numerous fibrillar centres, mainly of homogeneous type, and occasionally nucleolar vacuoles could be observed. The cytoplasm was very rich in ribosomes and in various organelles like plastids and endoplasmic reticulum; cytoplasmic vacuoles of different sizes were also present (Fig. 3B).

Anti-MAPK antibodies provided specific labelling on the cytoplasm and on particular subcompartments of the nucleus of proliferating plant cells. The labelling was abundant in the nucleus, apparently greater than in the cytoplasm where gold particles were found dispersed throughout large cytosolic areas (Fig. 3D). A very low background level was observed on both cytoplasmic vacuoles or cell walls. In the nucleus, the interchromatin region showed the highest intensity of labelling. Only very few gold particles were seen on condensed chromatin masses (Fig. 3D); the low labelling density on chromatin seemed to be similar to that showed by cytoplasmic vacuoles, and represents background.

EDTA regressive staining, preferential for ribonucleoproteins (RNPs) was performed after the immunogold labelling. This staining bleaches the condensed chromatin masses whereas RNP structures, like the nucleolus and fibres and granules of the interchromatin region (IR), appear highly electron dense (Fig. 4A). The combination of the EDTA staining with the immunogold labelling for anti-MAPK shows that gold particles are localized on stained fibrillar structures of the IR (Fig. 4B), the EDTA-positive granules being free of labelling.

Comparing the labelling pattern exhibited in the two cell types, quiescent and proliferating, more abundant anti-MAPK labelling was clearly recognized in the cycling cells, although the subcellular distribution of gold particles was similar, which demonstrated that the antigen was localized in the same cellular compartments in both plant cell types: namely the cytoplasm and nuclear interchromatin region.

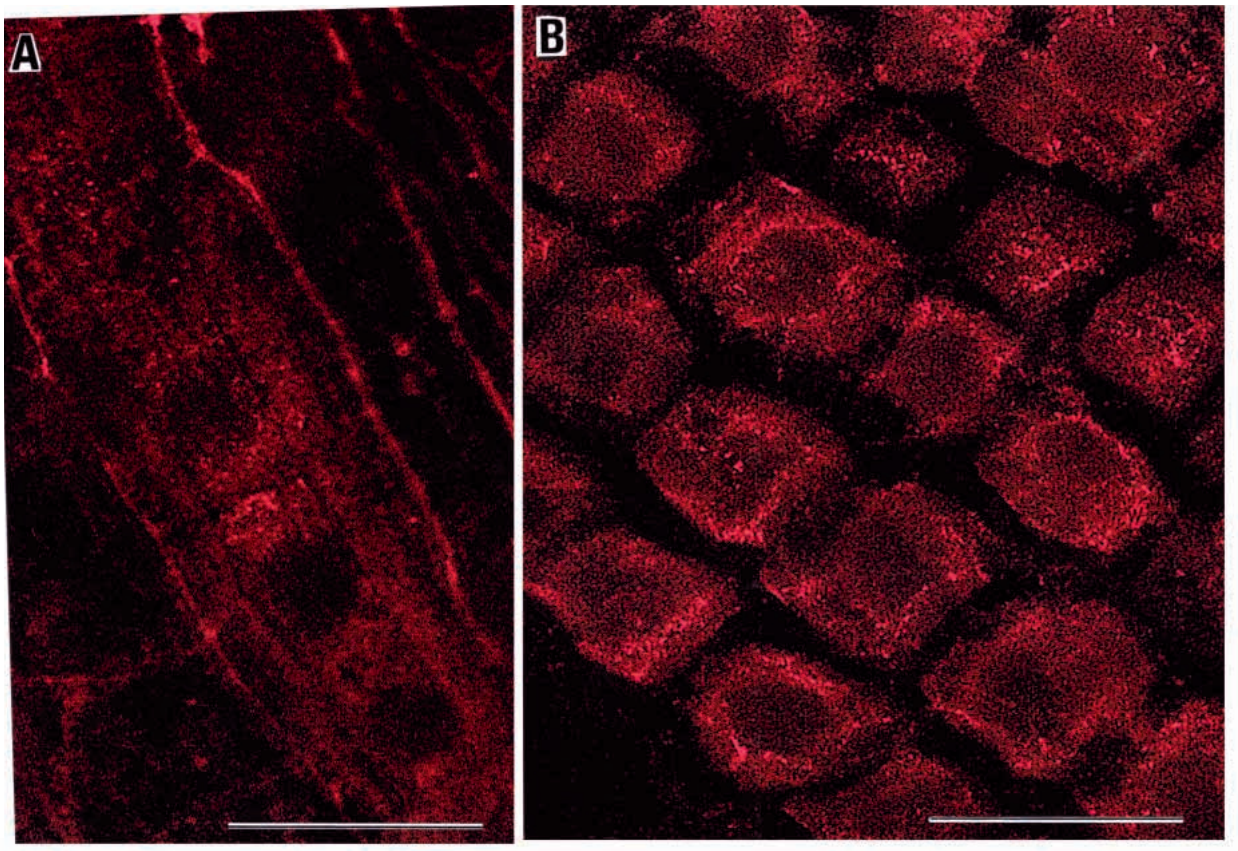

Fig. 2. Immunofluorescence with anti-MAPK in quiescent (A) and proliferating (B) onion root meristems. Confocal microscopy observations of 40 μm vibratome sections, the images represent projections of 10-15 optical sections. Positive immunofluorescence is observed in the cytoplasm of both cell types. A weak fluorescence can be discerned as heterogeneous signal in some nuclei of the proliferating cells. Bars, 100 μm.

Pepper vacuolate microspores

These cells were characterized by the presence in the cytoplasm of an extremely large vacuole, which pushes the nucleus to the periphery of the cell, near the cell wall (Fig. 5A). The cytoplasm was reduced to a thin layer around the nucleus and the vacuole. The main parts of the well-structured special pollen wall, the exine, was already formed at this developmental stage and could be seen surrounding the microspores. The nucleus showed a very decondensed chromatin pattern with scarce and small chromatin patches, mainly localized at the nuclear periphery, and large areas corresponding to the interchromatin region which contained numerous fibres and granules of different structure and composition (Fig. 5A).

Anti-MAPK labelling was found on the cytoplasm and the nucleus of the vacuolate microspores, mainly over the large interchromatin space of these cells, the small-condensed chromatin masses being free of labelling (Fig. 5C). The rest of the cellular structures, like cytoplasmic vacuole or pollen wall, were almost free of labelling (Fig. 5C). Interestingly, the labelling in the vacuolate microspore nucleus seemed to be particularly abundant in comparison with the other plant cell types studied.

Controls performed avoiding the first antibody during incubation were completely negative in all tested samples, no significant labelling being observed in any cellular compartment (data not shown).

Quantitative evaluation of immunogold labelling

A statistical analysis of immunogold labelling was done to assess the density of gold particles over the positive labelled areas in cycling and quiescent root cells.

The labelling density, determined as the number of gold particles/μm^2, was calculated in various cellular subcompartments: cytoplasm, condensed chromatin, interchromatin region and cytoplasmic vacuoles.

The scarce labelling density found in the vacuoles was considered as an internal control of the level of background, which showed a mean value of 6.60 particles/μm^2. The labelling density value in the condensed chromatin masses was near the estimated unspecific binding showed by the antibody, therefore, labelling on chromatin was considered as background. Values in condensed chromatin and cytoplasmic vacuoles were similar in both cell types.

The results are represented in the histograms in Fig. 7. Different densities of labelling are observed among the cellular subcompartments analyzed and the two different cell types studied. Labelling with anti-MAPK antibodies was more abundant in the nucleus than in the cytoplasm in both quiescent and cycling cells, the interchromatin region being the nuclear subcompartment with the highest and significant labelling density.

The comparison of anti-MAPK labelling density between quiescent and cycling cells clearly showed differences between the two cell types in the same cellular compartments (Fig. 7).

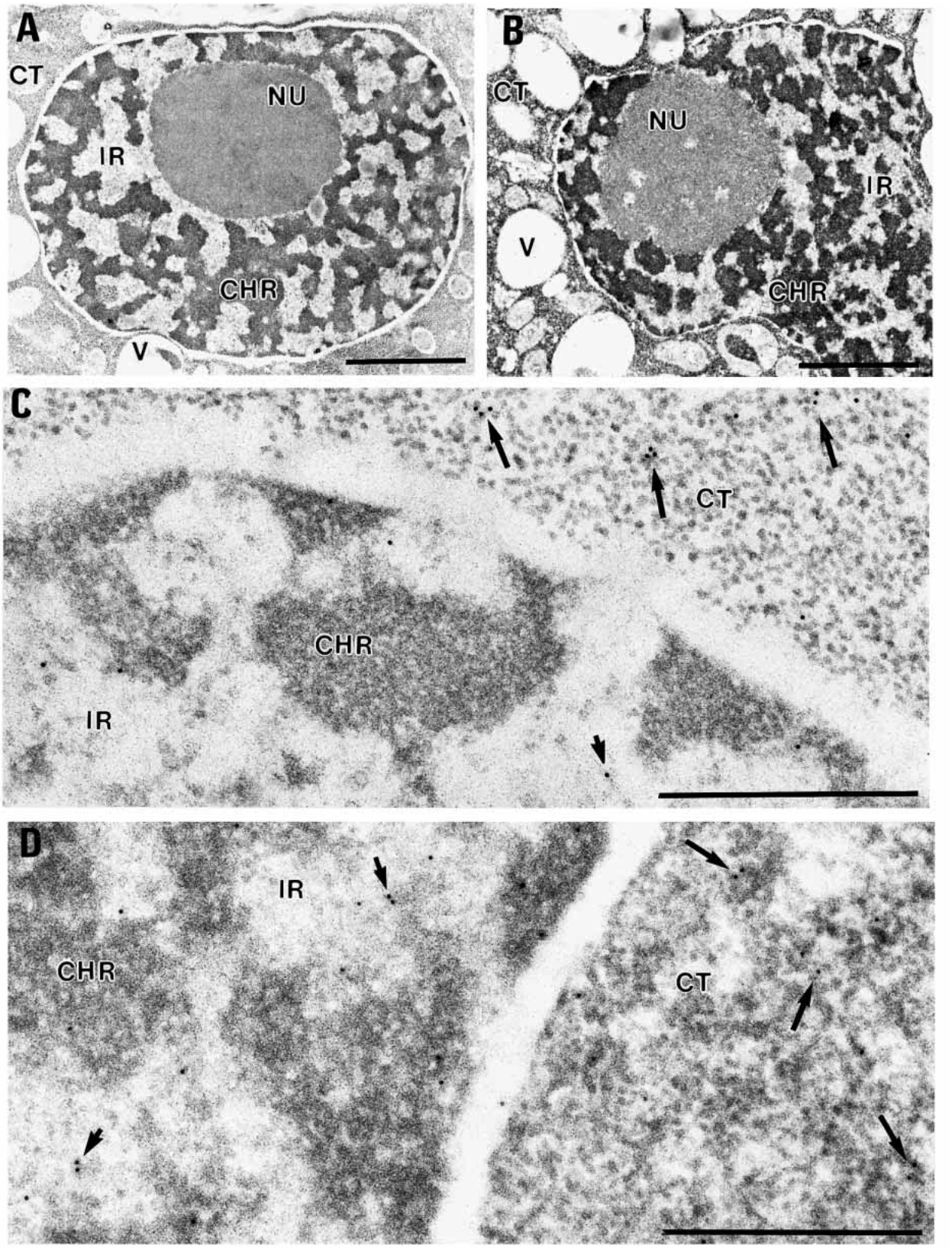

Fig. 3. Anti-MAPK immunogold labelling on quiescent and proliferating root meristematic cells. Formaldehyde fixation, PLT dehydration, methylation-acetylation cytochemistry en bloc, Lowicryl embedding. (A) Quiescent root cell, (B) proliferating root cell, (C and D) anti-MAPK immunogold labelling, (C) quiescent cell, (D) proliferating cell. Gold particles (arrows) are decorating cytoplasmic areas (CT) and, in the nucleus, the interchromatin region (IR). Cytoplasmic vacuoles (V) appear mainly free of labelling, as well as condensed chromatin masses (CHR). NU, nucleolus. Bars: 1 μm (A and B); 0.5 μm (C and D).

Cycling cells displayed higher density values than quiescent ones. Differences were found in the cytoplasm and the interchromatin region, which showed a much higher labelling density in cycling than in quiescent cells.

In situ hybridization

In situ hybridization (ISH) with digoxigenin-labelled RNA probes was performed on meristematic quiescent and proliferating onion root cells and pepper vacuolate microspores. RNA/RNA hybrids were subsequently visualized at the ultrastructural level using anti-digoxigenin antibodies conjugated to colloidal gold.

ISH results showed the presence of MAPK transcripts in all the plant cell types studied. In quiescent root cells, the hybridization signal was mostly observed in the cytosol, the nucleus showing only scarce labelling (Fig. 6A). In the nucleus, gold particles occasionally appeared in the interchromatin region. The ISH reaction seemed to be very specific as illustrated by the very low level of unspecific signal observed on cytoplasmic vacuoles and condensed chromatin masses (Fig. 6A). Gold particles over cytosolic areas frequently appeared as linear arrays of 2-4 particles (Fig. 6A).

The ISH signal observed in cycling cells seemed to be much higher than that found in quiescent cells. This was true for the cytoplasm, where the highest labelling was found, and also for the interchromatin region (Fig. 6B). The cytoplasm was completely decorated by gold particles in proliferating cells (Fig. 6B). A hybridization signal was more frequently found on the nuclei of proliferating cells than of quiescent cells.

MAPK transcripts were also observed in vacuolate microspores. Gold particles decorated the thin layer of cytoplasm at the periphery of the cell, the large cytoplasmic vacuole being mainly free of labelling (Fig. 5B). RNA MAPK probes also labelled the nucleus. The large interchromatin region of the vacuolate microspore showed abundant hybridization signals, which frequently appeared as lines of 2-3 gold particles (arrowheads in Fig. 5B).

Controls performed using the digoxigenin-labelled sense RNA probe did not show labelling to any cellular subcompartment in all the cell types studied (Fig. 6C and D).

Quantitative evaluation of in situ hybridization results

The number of gold particles/μm^2 was estimated in various

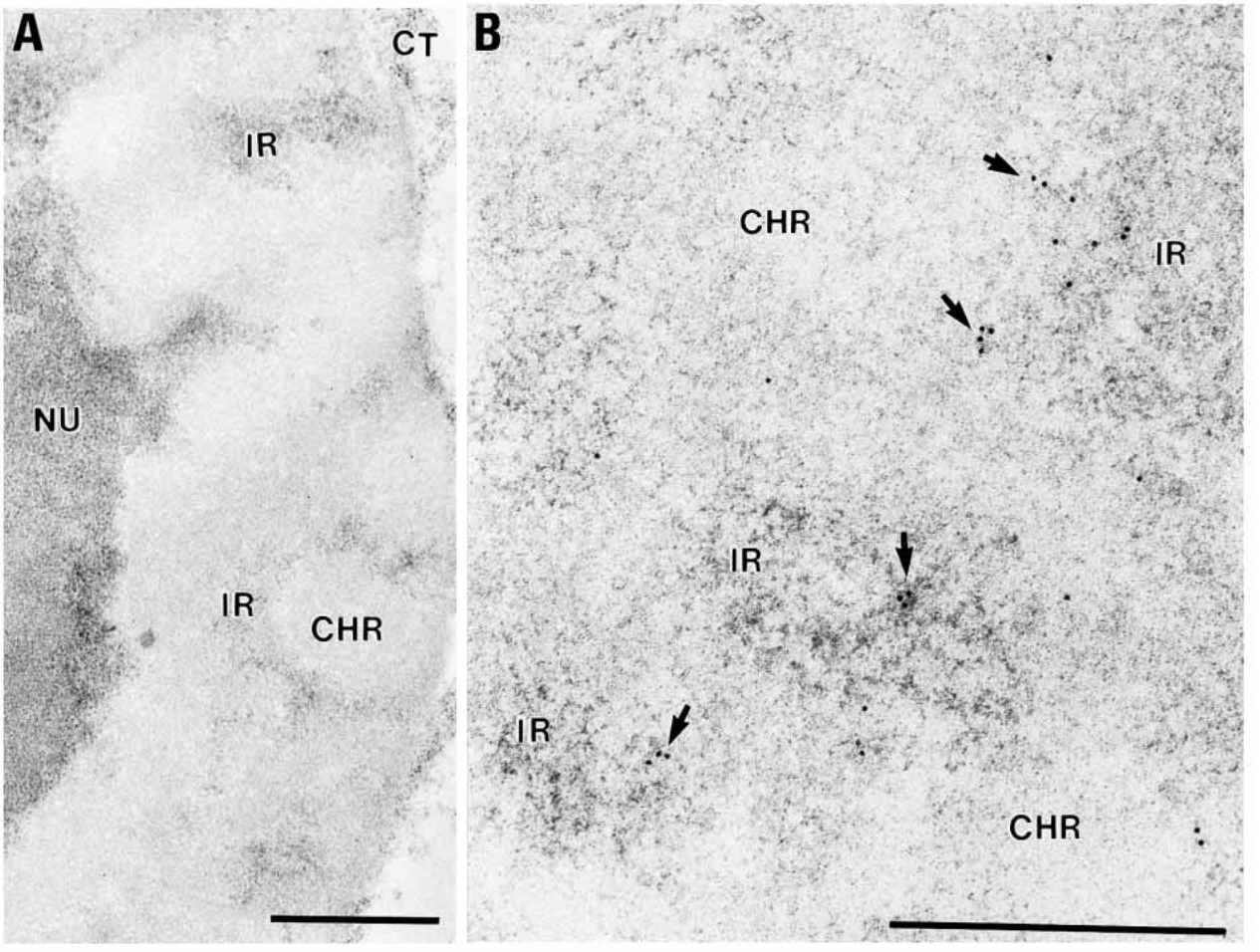

Fig. 4. Anti-MAPK immunogold labelling combined with EDTA staining for ribonucleoproteins. Proliferating onion root meristematic cells. Formaldehyde fixation, PLT dehydration, Lowicryl embedding. (A) General view of a nuclear region. Condensed chromatin masses (CHR) appear bleached whereas the nucleolus (NU) and structures in the interchromatin region (IR) are contrasted. (B) Nuclear region at a higher magnification. Gold particles (arrows) are localized on EDTA-stained fibrillar structures of the IR. Bleached chromatin patches (CHR) show no labelling. CT, cytoplasm. Bars, 0.5 μm.

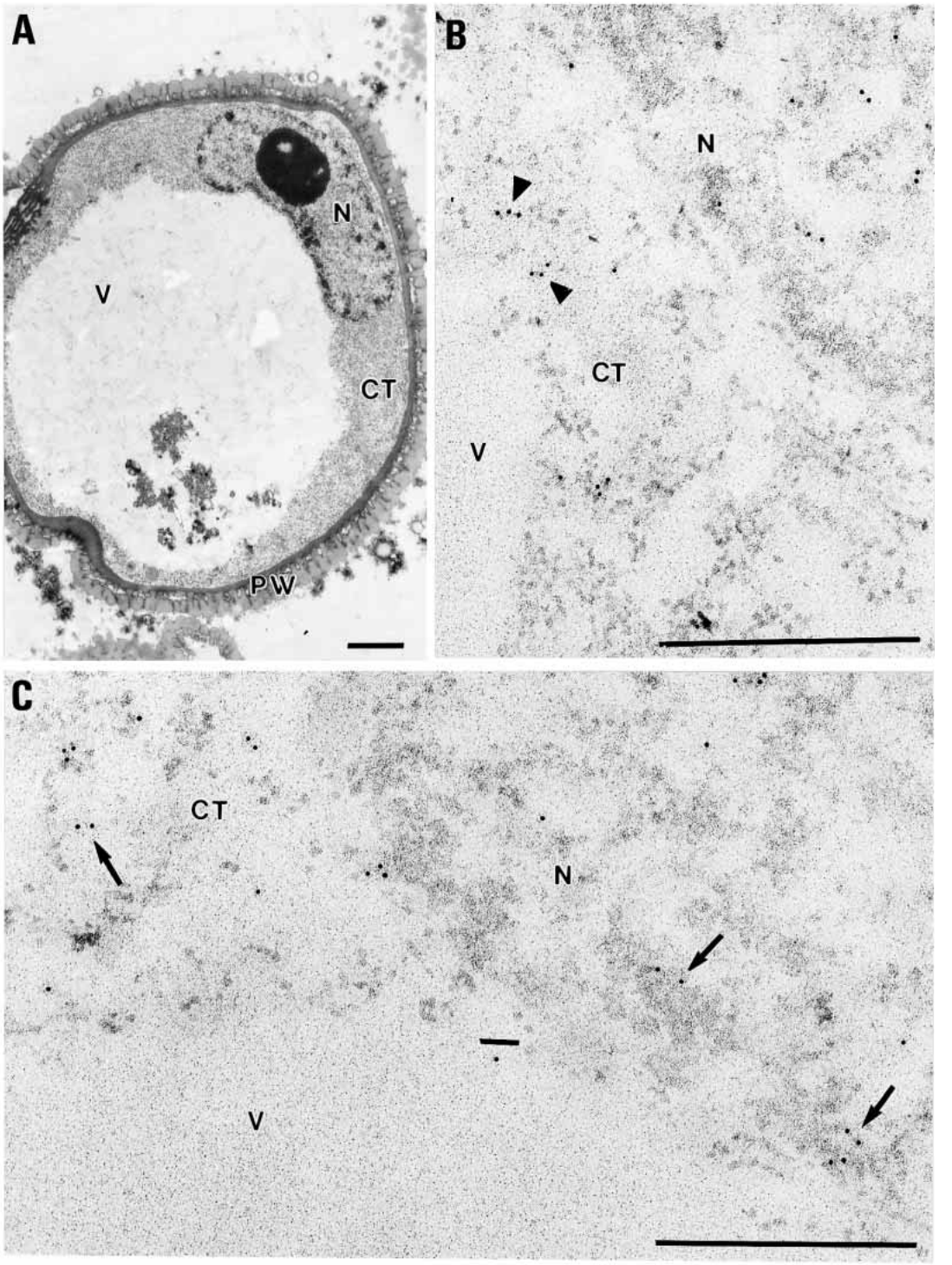

Fig. 5. Anti-MAPK immunogold labelling and in situ hybridization in vacuolate microspores. Formaldehyde fixation, PLT dehydration, methylation-acetylation cytochemistry en bloc, Lowicryl embedding. (A) Vacuolate microspore at low magnification, a large cytoplasmic vacuole (V) occupies the major part of the cellular volume, only a thin layer of cytoplasm (CT) is surrounding the inner part of the pollen cell wall (PW). The nucleus (N) is located at the periphery. (B) MAPK in situ hybridization with anti-sense probes; gold particles are decorating the cytoplasm (CT) and the nucleus (N), linear arrays of particles (arrowheads) are frequently observed. (C) Anti-MAPK immunogold labelling, gold particles (arrows) are localized over the thin layer of cytoplasm (CT) and dispersed throughout the nucleus (N) which displays a very decondensed pattern of chromatin. Other cellular subcompartments, as the large cytoplasmic vacuole (V), do not show any labelling. Bars: 1 μm (A); 0.5 μm (B and C).

cellular subcompartments (condensed chromatin, interchromatin region, cytoplasm and cytoplasmic vacuoles) on cycling and quiescent onion root meristems. The results of the quantitative evaluation of the ISH experiments are shown in Fig. 8.

In both cell types evaluated, the distribution of labelling density values was similar among the cellular subcompartments studied. The cytoplasm was the cellular compartment showing the highest hybridization signal. A much lower, but clearly significant, labelling density was found in the interchromatin region. Very low levels of background (average of 1.59 particles/μm^2) were estimated as labelling density in cytoplasmic vacuoles and condensed chromatin.

A comparison of ISH results in proliferating and quiescent cells shows a rather higher hybridization signal in cycling cells than in quiescent cells. Labelling density in cytosol was more than double the density in cycling cells. In the nucleus, labelling density on the interchromatin region of proliferating cells was also much higher than that of quiescent cells (Fig. 8).

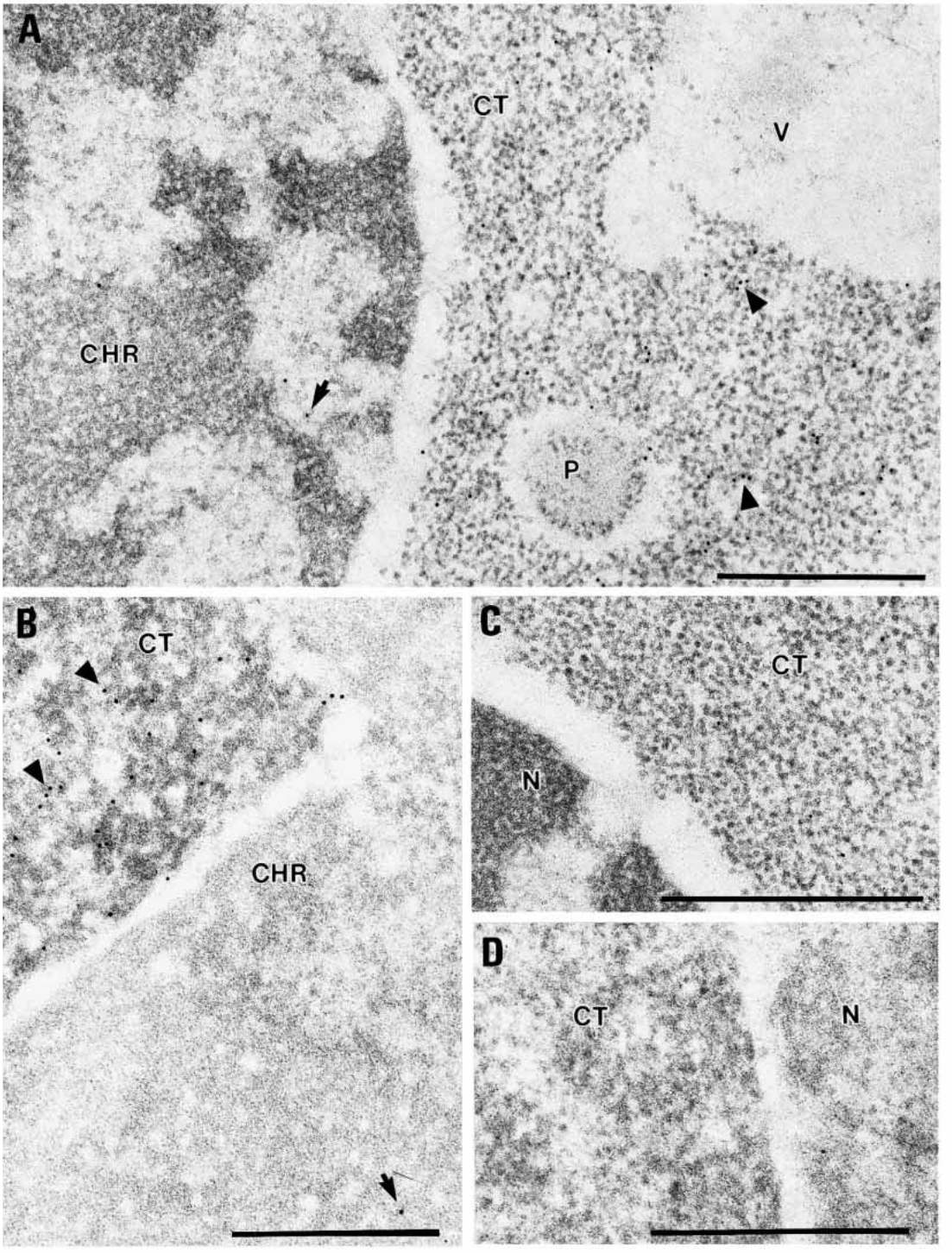

Fig. 6. In situ hybridization for localization of MAPK homologue mRNAs in meristematic root cells. Formaldehyde fixation, PLT dehydration, Methylation-Acetylation cytochemistry en bloc, Lowicryl embedding. (A) Anti-sense probe, quiescent cell. Some hybridization signal (arrowheads) is observed in the cytoplasm (CT), a very few gold particles (arrow) are localized in the interchromatin region (IR). (B) Anti-sense probe, cycling cell. High labelling density is observed in the cytoplasm (CT), 2-3 particles forming linear arrays (arrowheads) are frequently found. Some gold particles (arrows) are also observed on the interchromatin region (IR) of the nucleus. No significant labelling is observed on cytoplasmic vacuoles (V) and condensed chromatin (CHR). (C and D) Control experiments with the sense probe in quiescent (C) and proliferating (D) cells, no gold labelling is observed. P, plastid. Bars, 0.5 μm.

DISCUSSION

Homologues of MAPK have been found and cloned in various plant species (Wilson et al., 1993, 1995; Duerr et al., 1993; Decroocq-Ferrant et al., 1995; Jonak et al., 1993, 1994, 1995, 1996; Mizoguchi et al., 1993, 1994; Stafstrom et al., 1993). This paper reports for the first time since our preliminary report (Préstamo et al., 1997), the ultrastructural in situ localization of MAPKs and their transcripts in plant cell types displaying different states of activity.

Immunocytochemical studies have shown that a conserved epitope of MAPK homologues in plants is present in root meristematic cells, independent of the state of cell activity, and also in vacuolate microspores. We have used an antibody raised against a peptide synthesized from a short region of a tobacco Ntf6 clone (Wilson et al., 1995; Calderini et al., 1998). This antibody recognizes a single band on protein extracts from onion root meristems whose molecular mass is in the range (43-45 kDa) for the deduced protein product of the Ntf6 clone (Wilson et al., 1995; Calderini et al., 1998). This indicates the

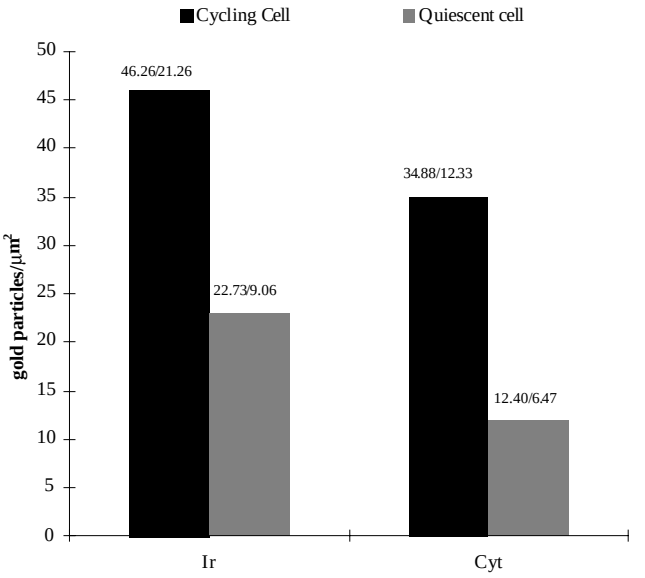

Fig. 7. Histogram showing the quantitative evaluation of anti-MAPK labelling density (number of gold particles/µm^2, in ordinates) over different subcellular compartments of root meristematic cells. Dark bars represent data from cycling cells, and light bars correspond to the quiescent ones. Data show the average and standard deviation, Interchromatin region in the nucleus, Ir; cytoplasm, Cyt.

presence of a MAPK homologue protein in the plant species used in this study, and illustrates the specificity of the signal observed in the in situ localizations.

Considering the situation observed in other species, such as tobacco, alfalfa or *Arabidopsis*, one could also assume the presence of several distinct MAPK also in the species used in the present work, namely pepper and onion. The question arises, therefore, as to which of these MAPKs have been detected in the immunolocalization and in situ hybridization experiments. Although the definitive answer should await the isolation and characterization of (all) pepper and onion MAP kinases, the specificity of the antibody used in the present study and the immunoblotting results strongly suggest that it is a close homologue of the tobacco Ntf6 MAP kinase (Wilson et al., 1995). This antibody was raised against a synthetic peptide derived from the carboxy-terminal sequence of Ntf6, corresponding to its last 12 amino acids; it has been shown to be specific for the recombinant Ntf6 protein, in western blots and immunoprecipitation experiments, not crossreacting with the other two tobacco MAP kinases, Ntf3 and Ntf4, isolated by us (unpublished results). Similarly, polyclonal antibodies prepared against synthetic peptides derived from the last amino acids of three different alfalfa MAP kinases, are highly specific for their corresponding recombinant protein (Jonak et al., 1996; Bögre et al., 1997). Although the possibility cannot be completely excluded, it is extremely unlikely that the antibodies could have cross-reacted with another, unrelated protein. The conservation in different species of the epitope recognized by the antibody used is suggestive of the presence in pepper microspores and onion root cells of a functional homologue of the tobacco Ntf6 MAP kinase.

The pattern of distribution of the protein is similar in all plant cell types studied: it is localized in the cytoplasm and the nucleus. The presence of MAPK in the cytoplasm and the nucleus has been reported in a few studies in mammalian cells by immunofluorescence (Shangera et al., 1992; Chen et al., 1992). In HeLa cells, some MAPK immunofluorescence was reported on the perinuclear area (Chen et al., 1992) Our results show an immunofluorescence pattern in agreement with these previous reports in animal cells. Concerning plant cells, in a very recent paper, a diffuse immunofluorescence staining was found in suspension tobacco cells with anti-Ntf6 MAPK antibodies (Calderini et al., 1998), the same antibody which is used in the present work; however, the MAPK immunofluorescence data shown in our paper provide new information on the subcompartmentalization of this protein in plant cells, i.e. it is present in the nucleus and in the cytoplasm. The higher labelling density of the perinuclear area of the cytoplasms on projections of consecutive confocal sections of anti-MAPK labelled onion root meristems was observed, but the functional significance of this localization has not yet been established.

The results described here at the ultrastructural level correlate with the immunofluorescence signals; they enabled the precise localization of the subcellular distribution of the antigen. It appeared in the cytoplasm and in the nucleus, mainly in the interchromatin region, the nuclear area among the condensed chromatin masses. Immunofluorescence assays with the same anti-MAPK antibodies in tobacco cells revealed no positive signal on DAPI-stained nuclear regions, i.e. chromatin (Calderini et al., 1998). When EDTA staining for RNPs was combined with anti-MAPK immunogold labelling, EDTA staining revealed that MAPK labelling is associated with ribonucleoprotein fibrillar structures of the interchromatin region, the subnuclear domain where chromatin fibres, transcripts in processing, and the machinery of replication,

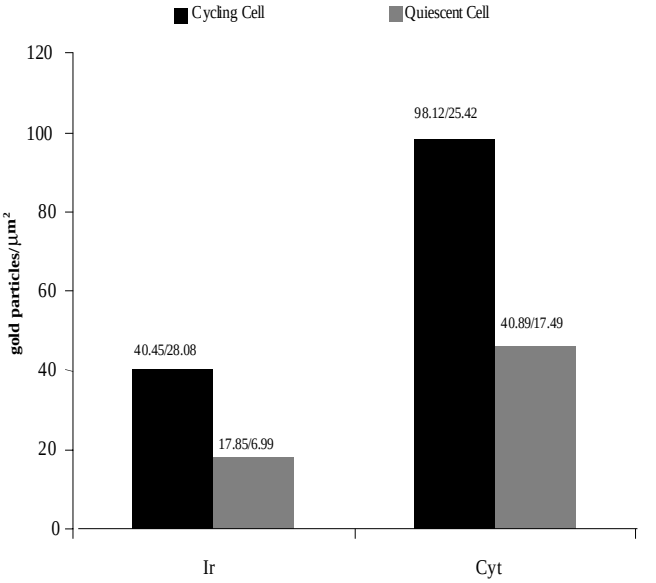

Fig. 8. Histogram showing the quantitative evaluation of labelling density (number of gold particles/µm^2, in ordinates) over different subcellular compartments of root meristematic cells after in situ hybridization for MAPK homologue expression. Dark bars represent data from cycling cells, and light bars correspond to the quiescent ones. Data show the average and standard deviation. Interchromatin region in the nucleus, Ir; cytoplasm, Cyt.

transcription and processing have been localized (Testillano et al., 1991, 1993, 1994, 1995a; González-Melendi et al., 1996a, 1998; Olmedilla et al., 1993). Proliferating and quiescent meristematic plant cells (Risueño and Moreno-Díaz de la Espina, 1979) were studied in order to compare the presence and distribution of MAPK in relation to the proliferation or quiescent state of the cell. This quantitative study clearly showed that MAPK is more abundant in cycling than in quiescent cells of the same tissue and species. The involvement of MAPK in the re-entry into the cell cycle is well documented in different eukaryotic cells (Meloche et al., 1992; Chen et al., 1992; Ruderman, 1993), and a possible role in cytokinesis in tobacco cells has been recently suggested for this MAPK (Calderini et al., 1998). The results presented here indicate that a greater MAPK presence is associated with the entry into proliferation and suggest that MAPK pathways might play a role in cell proliferation in plants as well as animals.

In animal cells, MAPKs have been shown to move from the cytoplasm to the nucleus, to phosphorylate transcription factors in association with the entry into mitosis of quiescent cells in serum-deprived cultures (Chen et al., 1992). The quantification of labelling density in the cytoplasm and nuclei of quiescent and proliferating root meristematic cells shows a significant increase in labelling in the nucleus of cycling cells compared to the quiescent ones. A greater presence of MAPK antigen is also observed in the cytoplasm of proliferating cells. From these results, entry into the cell cycle might be associated with translocation of MAPK into the nucleus but an increase in protein synthesis would occur, since labelling density also increases in the cytoplasm. Further studies concerning the activation of MAPK and its effects on protein distribution in plant cells are needed to clarify this point.

The immunogold results also show the presence of MAPK in the cytoplasm and nucleus of pepper vacuolate microspores. Expression of MAPK plant homologue genes has been reported in pollen grains of tobacco (Jonak et al., 1993; Wilson et al., 1995, 1997), but there was no data about the in situ localization of these proteins at any stages during pollen development. Vacuolate microspores in this plant species have been reported to be responsive to stress treatments inducing embryogenesis, giving rise to a haploid embryo (González-Melendi et al., 1995, 1996a,b; Testillano et al., 1995b). Further comparative work on the immunolocalization and distribution of MAPK antigens during the inductive treatment for embryogenesis would shed light on the involvement of MAPK modules in the signal transduction of stress to trigger embryogenesis in the microspore.

Very little information is available about MAPK expression in plants. The reported results show the presence of MAPK transcripts in all plant cell types studied, but it is much lower in quiescent cells compared to proliferating cells. The quantitative study and the controls with the sense probes show the specificity of the in situ hybridization (ISH) assay. Very low background levels were found on cellular subcompartments expected to be negative (such as cytoplasmic vacuoles or condensed chromatin), and almost no signal was observed in parallel experiments with labelled-sense probes. The antisense RNA probe used in the in situ hybridization experiments is not specific for Ntf6 (or its homologues). This probe corresponds to part of the conserved catalytic domain of MAP kinases and, under the conditions used, should cross-hybridize with all MAP kinase mRNAs. Since it is not known whether the Ntf6-like genes are the only MAP kinase genes expressed in microspores or onion root cells, the signals detected may correspond to the pool of all MAP kinase transcripts present in the samples. Nevertheless, the results of the in situ hybridization clearly shows the expression of (a) MAP kinase gene(s), and correlate well with the immunolocalization experiments, supporting their conclusions.

The different densities of labelling, revealed by the quantitative study of ISH signals, strongly suggest that a higher level of MAPK expression occurs in proliferating cells, although there might be some basal messenger presence and/or expression in the quiescent ones. These data are in relation to previous reports in alfalfa, where a MAPK homologue gene showed different expression levels at different cell cycle periods, G_1 phase showing much less expression than S and G_2 (Jonak et al., 1993). G_1 represents a phase of low cellular activity when compared with the rest of the cycle phases. In this study, different cell cycle phases were not analyzed separately but the results with the ISH are in relation to those in alfalfa since low levels of expression are associated with low cellular activity (as in quiescent cells). Moreover, the data would indicate a role played by MAPK expression in the entry into proliferation in plant cells.

In summary, the study on the localization of MAPKs and their transcripts in cycling and quiescent cells permits the extrapolation of a functional role for MAPKs in terms of proliferation, as the highest densities of gold labelling have been found on cycling cells. Interestingly, when ultrastructural immunogold and in situ hybridization were applied to pepper vacuolate microspores, a high intensity of gold labelling was observed. Vacuolate microspores are intermediate stages during pollen development and throughout microsporogenesis the cell is being prepared for an unequal mitotic division producing the bicellular pollen grain. We have previously reported (González-Melendi et al., 1995, 1996a, 1996b; Testillano et al 1995a) that under stress conditions, vacuolate microspores can deviate from their normal process of development towards an embryonic pathway. Under those conditions, the microspore nucleus divides symmetrically and further proliferates to multicellular structures. Work is now in progress to determine a possible role for MAPK in reprogramming vacuolate microspores towards embryogenesis.

We thank Dr M. T. Rejas (CBMSO) for her excellent technical collaboration, Ms M. A. Ollacarizqueta for her advice with the confocal microscope (Service of Confocal Microscopy of C.I.B.) and her useful help in other tasks, the help and advice for the quantitative study of Dr J. Renau-Piqueras (La Fe Hospital, Valencia), the expert photographic work of Ms V. Muñoz and M. Fontela (CIB) and Mr J. A. Perez-Gracia (CBMSO), the English style checking of Ms B. Ligus-Walker. This work was supported by grants from the European Union 4 BIOTECH Program SIME project (BIO4-CT96-0275), and Spanish DGICYT PB95-0133; it has also been partially supported by the Spanish-Austrian Joint Project CSIC/Vienna University to M.C.R. and E.H.B. (HU97-0033).

REFERENCES

Blumer, K. K. and Johnson, G. L. (1994). Diversity in function and regulation of MAP kinase pathways. *Trends Biochem. Sci.* **19**, 236-240.

Bögre, L., Ligterink, W., Meskiene, Y., Barker, P. J., Heberle-Bors, E.,

Huskisson, N. S. and Hirt, H. (1997). Wounding induces the rapid and transient activation of specific MAP kinase pathway. *Plant Cell* **9**, 75-83.

Calderini, O., Bögre, L., Vicente, O., Binarova, P., Heberle-Bors, E. and Wilson, C. (1998). A cell cycle regulated MAP kinase with a possible role in cytokinesis in tobacco cells. *J. Cell Sci.* **111**, 3091-3100.

Chen, R. H., Sarnecki, CH. and Blenis, J. (1992). Nuclear localization and regulation of erk- and rsk-encoded protein kinases. *Mol Cell Biol*. **12,** 915-927.

Davis, R. J. (1994). MAPKs: new JNK expands the group. *Trends Biochem Sci.* **19**, 470-473.

Decroocq-Ferrant, V., Decroocq, S., Van-Went, J., Schmidt, S and Kreiss, M. (1995). A homologue of the MAP/ERK family of protein kinases genes is expressed in vegetative and in female reproductive organs of *Petunia hybrida*. *Plant Mol. Biol*. **27**, 339-350.

Duerr, B., Gawienowski, M., Ropp, T and Jacobs, T. (1993). MsERK1: a mitogen-activated protein kinase from a flowering plant. *Plant Cell* **5**, 87-96.

González-Melendi, P., Testillano, P. S., Ahmadian, P., Fadón, B., Vicente, O. and Risueño, M. C. (1995). In situ characterization of the late vacuolate microspore as a convenient stage to induce embryogenesis in *Capsicum*. *Protoplasma.* **187**, 60-71.

González-Melendi, P., Testillano, P. S., Ahmadian, P., Fadón, B. and Risueño, M. C. (1996a). New in situ approaches to study the induction of pollen embryogenesis in *Capsicum annuum L*. *Eur. J. Cell Biol*. **69**, 373-386.

González-Melendi, P., Testillano, P. S., Préstamo, G., Fadón, B. and Risueño, M. C. (1996b). Cellular characterization of key developmental stages for pollen embriogenesis induction. *Int. J. Dev. Biol*. Suppl **1**, 127-128.

González-Melendi, P., Testillano, P. S., Mena, C. G., Müller, S., Raska, I. And Risueño, M. C. (1998). Histones and DNA ultrastructural distribution in plant cell nucleus: a combination of immunogold and cytochemical methods. *Exp. Cell Res.* **242**, 45-59.

Herskowitz, Y. (1995). MAP kinase pathways in yeast: for mating and more. *Cell* **80**, 187-197.

Hirt, H. (1997). Multiple roles of MAP kinase in plant signal transduction. *Trends Plant Sci.* **2**, 11-15.

Jonak, C., Páy, A., Bögre, L., Hirt, H. and Heberle-Bors, E. (1993). The plant homologue of MAP kinase is expressed in a cell cycle-dependent and organ-specific manner. *Plant J*. **3**, 611-617.

Jonak, C., Heberle-Bors, E. and Hirt, H. (1994). MAP kinases: Universal multi-purpose signaling tools. *Plant Mol. Biol*. **24**, 407-416.

Jonak, C., Kiegerl, S., Lloyd, C., Chan, J. and Hirt, H. (1995). MMK2, a novel alfalfa MAP kinase, specifically complements the yeast MPK1 function. *Mol. Gen. Genet.* **248**, 686-694.

Jonak, C., Kiegerl, S., Ligterink, W., Barker, P. J., Huskisson, N. S. and Hirt, H. (1996). Stress signaling in plants: A mitogen-activated protein kinase pathway is activated by cold and drought. *Proc. Nat. Acad. Sci. USA* **93**, 11274-11279.

Marshall, C. J. (1994). MAP kinase kinase kinase, MAP kinase kinase, and MAP kinase. *Curr. Opin. Genet. Dev.* **4**, 82-89.

Meloche, S., Seuwen, K., Pages, G., Pouyssegur, J. (1992). Biphasic and synergistic activation of $p44^{mapk}$ (ERK1) by growth factors: correlation between late phase activation and mitogenicity. *Mol. Endocrinol*. **6**, 845-854.

Mena, C. G., Testillano, P. S., González-Melendi, P., Gorab, E. and Risueño, M. C. (1994). Immunoelectron microscopy of RNA combined with nucleic acid cytochemistry in plant nucleoli. *Exp. Cell Res.* **212**, 393-408.

Mizoguchi, T., Hayashida, N., Yamaguchi-Shinozaki, K., Kamada, H., Shinozaki, K. (1993). ATMKs: a family of plant MAP kinases in *Arabidopsis thaliana. FEBS Lett*. **336**, 440-444.

Mizoguchi, T., Gotoh, Y., Nishida, E., Yamaguchi-Shinozaki, K. Hayashida, N., Iwasaki, T., Kamada, H. and Shinozaki, K. (1994). Characterization of two cDNAs that encode MAP kinase homologues in *Arabidopsis thaliana* and analysis of the possible role of auxin in activating such kinase activities in cultured cells. *Plant J*. **5**, 111-122.

Olmedilla, A., Testillano, P. S., Vicente, O., Delseny, M. and Risueño, M. C. (1993). Ultraestructural rRNA localization in plant cell nucleoli. RNA/RNA in situ hybridization, autoradiography and cytochemistry. *J. Cell Sci*. **106**, 1333-1346.

Payne, D. M., Rossomando, A. J., Martino, P., Erikson, A. K., Her, J. H., Weber, M. J. and Sturgill, T. W. (1991). Identification of the regulatory phosphorylation sites in pp42/mitogen-activated protein kinase (MAP kinase). *EMBO J*. **10**, 885-892.

Pelech, S. L. and Shanghera, J. S. (1992). Mitogen-activated protein kinases: versatile transducers of cell signaling. *Trends Biochem Sci*. **17**, 233-238.

Posada, J. and Cooper, J. A. (1992). Requirements for phosphorylation of MAP kinase during meiosis in Xenopus oocytes. *Science* **255**, 212-215.

Préstamo, G., Testillano, P. S., González-Melendi, P., Vicente, O. and Risueño, M. C. (1997). Ultrastructural localization of MAPK and their transcripts in meristematic plant cells and developing pollen grains. In *Proceeding of the XVIII National Meeting of the Spanish Society for EM*, April 1997 (ed. A. Baró), pp. 3-4. Toledo.

Risueño, M. C. and Moreno-Diaz de la Espina, S. (1979). Ultraestructural and cytochemical study of the nucleus of the dormant root meristematic cells. *J. Submicrosc. Cytol.* **11**, 85-95.

Risueño, M. C. and Testillano, P. S. (1994). Cytochemistry and immunocytochemistry of nucleolar chromatin in plants. *Micron* **25**, 331-360.

Ruderman, J. V. (1993). MAP kinase and the activation of quiescent cells. *Curr. Opin. Cell Biol*. **5**, 207-213.

Sanghera, J. S., Peter, M., Nigg, E. A. and Pelech, S. L. (1992). Immunological characterization of avian MAP kinases: Evidence for nuclear localization. *Mol. Biol. Cell* **3**, 775-787.

Schultz, J., Ferguson, B. and Sprague, G. F. (1995). Signal transduction and growth control in yeast. *Curr. Opin. Genet. Dev.* **5**, 31-37.

Stafstrom, J. P., Altschuler, M and Anderson, D. H. (1993). Molecular cloning and expression of a MAP kinase homologue from pea. *Plant Mol. Biol.* **22**, 83-90.

Testillano, P. S., Sánchez-Pina, M. A., Olmedilla, A., Ollacarizqueta, M. A., Tandler, C. J. and Risueño, M. C. (1991). A specific ultrastructural method to reveal DNA: The NAMA-Ur method. *J. Histochem. Cytochem.* **39**, 1427-1438.

Testillano, P. S., Sánchez-Pina, M. A., Olmedilla, A., Fuchs, J. P. and Risueño, M. C. (1993). Characterization of the interchromatin region as the nuclear domain containing snRNPs on plant cells. A cytochemical and immunoelectron microscopy study. *Eur. J. Cell Biol*. **61**, 349-361.

Testillano, P. S., Gorab, E. and Risueño, M. C. (1994). A new approach to Map transcription sites at the ultrastructural level. *J. Histochem. Cytochem.* **42**, 1-10.

Testillano, P. S., González-Melendi, P., Ahmadian, P., Fadón, B. and Risueño, M. C. (1995a). The immunolocalization of nuclear antigens to study the pollen developmental program and the induction of pollen embryogenesis. *Exp. Cell Res*. **221**, 41-54.

Testillano, P. S., González-Melendi, P., Ahmadian, P. and Risueño, M. C. (1995b). The methylation-acetylation method, an ultrastructural cytochemistry for nucleic acids compatible with immunogold studies. *J. Struct. Biol*. **114**, 123-139.

Wilson, C., Eller, N., Gartner, A., Vicente, O. and Heberle-Bors, E. (1993). Isolation and characterization of a tobacco cDNA clone encoding a putative MAP kinase. *Plant Mol. Biol*. **23**, 543-551.

Wilson, C., Anglmayer, R., Vicente, O. and Heberle-Bors, E. (1995). Molecular cloning, functional expression in Escherichia coli, and characterization of multiple mitogen-activated-protein kinases from tabaco. *Eur. J. Biochem*. **233**, 249-257.

Wilson, C., Voronin, V., Touraev, A., Vicente, O. and Heberle-Bors, E. (1997). A developmentally regulated MAP Kinase activated by hydration in tobacco pollen. *Plant Cell* **9**, 309-324.