ELSEVIER

Eur. J. Cell Biol. 83 (2004); 425–433
http://www.elsevier.de/ejcb

EJCB

Differential expression and cellular localization of ERKs during organogenic nodule formation from internodes of *Humulus lupulus* var. Nugget

Marta Sousa Silva[1)a], Ana Margarida Fortes[a], Pilar Sanchéz Testillano[b], Maria del Carmen Risueño[b], Maria Salomé Pais[a]

[a] Laboratory of Plant Biotechnology – ICAT, Campus of Science Faculty of Lisbon, Campo Grande, 1749–016 Lisboa, Portugal

[b] Plant Development and Nuclear Organization, Centro de Investigaciones Biológicas, CSIC, Velazquèz 144, E-28006 Madrid, Spain

Received May 19, 2003
Received in revised version June 4, 2004
Accepted June 4, 2004

Humulus lupulus var. Nugget; Nodule formation; ERK1/2; MAPKs; proliferation

The expression and subcellular localization of extracellular signal-regulated kinase 1 or 2 (ERK1/2) homologues (HLERK1/2) during the process of organogenic nodule formation in *Humulus lupulus* var. Nugget was studied using antibodies specific for ERK1 and ERK2, and for phosphorylated mitogen-activated protein kinases (MAPKs). The increase in HLERK levels, detected by Western blotting 12 hours after wounding suggests their involvement in response to the wounding treatment applied for morphogenesis induction. In dividing cambial cells, occurring in between 4 and 7 days after morphogenesis induction, as well as in dividing prenodular cells (15 days after induction) HLERK1 and/or 2 were localized in the nucleus. However, as soon as nodular cells start proliferating to form shoot meristems, HLERK1 and 2 were detected in the cytoplasm and not in the nucleus. The data reported account for a differential expression and activation of HLERK1 and HLERK2 throughout the process of nodule formation and plantlet regeneration. HLERK1 appears to be expressed in the stages of nodule formation and plantlet regeneration, playing a possible role in controlling cell proliferation and differentiation. HLERK2 may be induced as a response to reactive oxygen species (ROS) generated by wounding of internodes as its expression is reduced in liquid medium with less oxygen availability compared to solid medium. However, addition of a ROS inhibitor to the liquid medium does not result in a further decrease in the HLERK2 level.

[1)] **Corresponding author:** Dr. Marta Sousa Silva, Laboratory of Plant Biotechnology – ICAT, Campus of Science Faculty of Lisbon, Campo Grande, 1749–016 Lisboa, Portugal, e-mail: mfsilva@fc.ul.pt

Introduction

Signal transduction pathways are extremely diverse and have different strategies, in spite of having in common the activation of responses at the nuclear level by the modulation of gene expression. Throughout evolution common signal transduction pathways have been adopted, in particular phosphorylation/dephosphorylation reactions between proteins (Brunet, 1998). The ERK pathway, a conserved MAPK (mitogen-activated protein kinase) module along the eukaryote evolution (Stone and Walker, 1995), constitutes an example of signal transduction in cascade. In animal and yeast cells, the last members of this cascade, ERK1 (42 kDa) and ERK2 (44 kDa), have been involved in diverse cellular processes including control of cell growth, proliferation and differentiation (Marshall, 1995), cell cycle regulation and cell survival (reviewed by Meskiene and Hirt (2000)). These MAPKs are known to target many different substrates in the cytoplasm, such as cytoskeletal proteins, other kinases and phosphatases. When translocated to the nucleus they activate transcription factors and other nuclear proteins (Meskiene and Hirt, 2000; Schenk and Snaar-Jagalska, 1999). Studies on the human ERK cascade enabled the definition of a model for the involvement of these MAPKs in cell proliferation and differentiation. Depending on the duration of ERK activation, both proliferation and differentiation processes can be activated in the same cell (Marshall, 1995).

0171-9335/04/83/08-425 $30.00/0

Plant MAPKs show high homology with the ERK family of animal MAPKs (Meskiene and Hirt, 2000) and therefore, they are called plant extracellular regulated protein kinases (PERK) (Ligterink, 2000). The involvement of MAPKs in cell division, proliferation and differentiation in plants is not fully understood. The MMK3 in alfalfa and the Ntf6 in tobacco seem to be involved in cell cycle regulation (Bögre et al., 1999; Calderini et al., 1998). Their localization and activation during mitosis are similar to those reported for ERK1 and 2 in mammals (Meskiene and Hirt, 2000; Shapiro et al., 1998), suggesting that they play a similar role in mitosis. The ultrastructural in situ localization of a protein homologue of Ntf6 and its transcripts in onion cells and pollen grains, showed higher expression in proliferating cells than in quiescent cells (Prestamo et al., 1999). Recently, plant ERK1/2 homologues have been reported to increase in expression and to enter the nucleus during the pollen developmental program (Coronado et al., 2002).

Plant regeneration from organogenic nodules has been reported for some varieties of *Humulus lupulus* (Batista et al., 1996, 2000; Fortes and Pais, 2000), providing an important tool for large-scale micropropagation. The organogenic nodule formation from *Humulus lupulus* var. Nugget internodes is a developmental process that involves cell proliferation and differentiation, and seems to be induced by a wounding treatment (Fortes and Pais, 2000). This morphogenic process has been studied using several approaches, in order to better understand the induction and plantlet regeneration from these organogenic nodules. Wound-induced cellular events are mediated, in part, by the production of reactive oxygen species (ROS) (Leon et al., 2001) and involve the activation of mitogen-activated protein kinases (Seo and Ohashi, 2000). In hop, the production of ROS in wounded areas was reported. Internodes cultured in liquid organogenesis-inducing medium supplemented with N-acetyl-L-cysteine (NAC), a ROS inhibitor, showed little ROS accumulation 12 hours after culture initiation (Fortes et al., 2002a).

The expression and subcellular localization of ERK1/2 homologues and active phosphorylated MAPKs at different stages of organogenic nodule formation and plantlet development were studied, and their possible involvement in cell proliferation and differentiation is discussed. The expression of ERKs was also studied in the presence or absence of a ROS inhibitor and a possible involvement of ROS in the ERK1/2 pathway is addressed.

Materials and methods

Plant material

Internodal explants of *Humulus lupulus* var. Nugget in vitro growing plants were used to induce nodule formation as previously described by Fortes and Pais (2000). Micropropagation medium consisted of the MS macro- and micro-nutrients (Murashige and Skoog, 1962) with Adams vitamins, supplemented with 5 mg/l ascorbic acid, 0.2 mg/l BAP, 1 mg/l IBA, 20 g/l sucrose, and 7.8 g/l agar (Vaz Pereira, Lisbon, Portugal). Induction medium consisted of the MS complete medium supplemented with 30 mg/l cystein, 2 mg/l BAP, 0.05 mg/l IAA, 18 g/l sucrose, and 7.8 g/l agar. The pH was adjusted to 5.7–5.8. Cultures were maintained at 25 ± 2 °C under a 16 h photoperiod (35 μmol photons $\cdot$ $m^{-2} \cdot s^{-1}$) provided by cool-white Philips fluorescent tubes.

Antibodies

The polyclonal antibodies used were anti-ERK1/ERK2 and anti-phospho MAPK, which recognises the phosphorylated TEY sequence of the active MAPKs, (Cell Signaling, Beverly, MA).

Immunofluorescence assays and confocal microscopy

Induced explants collected at 0, 7, 15, 28, and 45 days were fixed in 4% (w/v) paraformaldehyde in phosphate-buffered saline (PBS: 137 mM NaCl; 3 mM KCl; 8 mM Na_2HPO_4; 3 mM KH_2PO_4; pH 7.3), at 4 °C overnight. After 3 washes for 5 min in PBS on ice, samples were cryoprotected by immersion in increasing sucrose concentrations: 1 hour on ice in 0.1 M sucrose in PBS, 3 hours at 4 °C under agitation in 1 M sucrose in PBS, and overnight at 4 °C under agitation in 2.3 M sucrose in PBS, embedded in tissue freezing medium (Jung, Leica Instruments GmbH) and frozen in carbonic ice (–80 °C). Thirty μm sections were obtained using a cryostat (Leica CM 1800) at –23 °C, and then placed on multiwell slides (ICN Biomedicals Inc., Ohio, USA) pre-treated with 3-aminopropyltriethoxysilane (Sigma, St. Louis, MO, USA) (Rentrop et al., 1986). Slides with sections were kept at –20 °C until use.

Immunofluorescence assays were performed mainly as described by Testillano et al. (1995) with slight modifications (Fortes et al., 2002b). Frozen glass slides carrying the sections were thawed at room temperature. After a wash with PBS, sections were permeabilized with 1% (w/v) cellulase (Onozuka R-10) in PBS for 40 min at room temperature, followed by three washes in PBS. A 20-min incubation with 0.5% (v/v) Triton X-100 in PBS, and three washes in PBS were performed before the incubation in 5% (w/v) BSA (Bovine Serum Albumin) in PBS for 5 min. The first antibody, being either anti-ERK1/ERK2 or anti-PhosphoMAPK, was applied undiluted, for 3 hours at 37 °C. Controls were performed by replacing the first antibody by PBS. After three washes in PBS, sections were incubated with the secondary antibody diluted 5:25 in 0.5% Evans Blue in PBS (Sigma), to remove autofluorescence of cell walls, for 45 min at room temperature, in the dark. Anti-rabbit-Alexa Fluor 488 (green fluorescence) (Molecular Probes, Leiden, The Netherlands) was used to detect the anti-ERK1/ERK2 as first antibody, and anti-rabbit Alexa Fluor 546 (red fluorescence) (Molecular Probes) was used when the first antibody was the anti-PhosphoMAPK. Sections were washed in PBS, stained with DAPI (4′-6-Diamidino-2-phenylindole), washed in bi-distilled water and finally mounted in Mowiol.

Confocal optical sections were collected using a BIO-RAD (Hemel Hempstead, UK) MRC-1024 confocal scanning head mounted on a ZEISS Axiovert 135 microscope (Zeiss, Jena, Germany). Images from sections with anti-ERK1/2 and DAPI were taken using an epifluorescence Zeiss Axioplan microscope with a CH250/A CCD photometry camera.

For double immunofluorescence assays, the antibody anti-ERK1/ERK2 was first used following the procedure previously described for the immunofluorescence assays, up to the incubation with the secondary antibody. The subsequent procedure was performed in darkness. After 3 washes in PBS, sections were incubated for 15 min in 1% (v/v) glutaraldehyde in PBS, and again washed once in PBS. The primary antibody anti-PhosphoMAPK was added after incubation with 50 mM ammonium chloride in PBS, 2 times for 20 min each (to block aldehyde groups as potential source of IgG unspecific binding), and 3 washes in PBS. The sections were incubated for 3 hours at 37 °C, as described earlier. After 3 washes in PBS, they were incubated for 45 min with the secondary antibody anti-rabbit-Alexa Fluor 546 diluted 5:25 in 0.5% Evans Blue in PBS. Sections were washed in PBS, then incubated for 5 min with 1% (v/v) glutaraldehyde and washed 2 times in PBS, prior to DAPI staining and final washes with double-distilled water. Confocal optical sections were then collected as described above.

Histological analysis

Samples were fixed overnight at 4 °C in 4% (w/v) paraformaldehyde in PBS, pH 7.3. After 3 washes (5 min each) in PBS, they were dehydrated through a graded series of ethanol. Then, samples were infiltrated and embedded overnight at room temperature in Historesin (Jung, Leica

Instruments GmbH, Heidelberg, Germany). Semithin sections were obtained using a LKB microtome (Leica, Vienna, Austria) and observed in a Zeiss Photomicroscope under phase contrast, for preliminary histological analysis.

Treatment with N-acetyl-L-cysteine (NAC)

Tubes containing micropropagated plants were filled with liquid nodule induction medium supplemented with 0.4 mM NAC. After 30 min, plants were transferred to Petri dishes containing the same medium. Internodes approximately 9 mm long were submitted to several incisions, while plants were kept immersed, and then transferred to Erlenmeyer flasks with the same induction medium. Controls were performed in both solid and liquid culture medium without inhibitor (Fortes et al., 2002a). Internodes were collected 12 h after induction and were used for immunoblotting studies.

Preparation of protein extracts

Induced explants collected at 7, 15, 28, and 45 days, and after 12 hours in induction medium with and without NAC, were frozen in liquid nitrogen and ground in a mortar. Explants at time 0 were obtained by immersing the entire plant in liquid nitrogen without touching it.

The extracts used for the Western blot with the anti-ERK1/2 antibody were prepared as described by Takabe et al. (1986). Briefly, 0.5 g of plant material was added to 5 ml of extraction buffer (50 mM Tris-HCl, pH 8.0, containing 0.2 M sucrose, 10 mM NaCl, 0.01% (w/v) sodium azide, 0.15 mM EDTA and the protease inhibitors 1 mM PMSF (Sigma), 1 μM leupeptin (Sigma) and 1 μM pepstatin A (Sigma)). After centrifugation for 10 min at 10000 rpm, the supernatant was filtered through Miracloth and precipitated overnight with 4 volumes of acetone (Merck, Germany) at 4 °C. Then, after a 15-min centrifugation at maximum speed, the pellet was vacuum dried and resuspended in an appropriate volume of a mixture of 100 mM Tris-HCl (pH 8.0), 10% (w/v) SDS and 5% (v/v) β-mercaptoethanol.

The extracts for the Western blot with the anti-phospho MAPK antibody were prepared using an extraction buffer, containing 25 mM HEPES, pH 7.5, 0.3 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.1% (v/v) Triton X-100, 20 mM β-glycerophosphate, 0.1 mM Na_3VO_4, and 1 mM PMSF. The tubes were vortexed and incubated at 4 °C for 10 min (Fabregat et al., 2000). After being centrifuged at 4 °C for 10 min, 13000 rpm, the supernatant was recovered.

The protein concentration in the extracts was quantified according to Bradford (1976) using BSA as standard.

SDS-PAGE and immunoblot analysis

Protein extracts from different stages of nodule formation (40 μg for the ERK1/2 immunoblot and 30 μg for the phospho-MAPK immunoblot) were denatured at 95 °C for 5 min in 10% (v/v) glycerol and 0.03% (w/v) bromophenol blue, and centrifuged for 15 min at maximum speed. Denatured protein extracts were subjected to 12% SDS-polycrylamide electrophoresis and thereafter transferred to Immobilon membranes (Millipore Corporation, Bedford MA/USA) using the Trans-Blot transfer cell (Bio-Rad). Prestained standard proteins (Bio-Rad, Hercules, CA, USA) were also loaded on the gel. Total proteins were then stained with Ponceau S solution (0.5% (w/v) Ponceau S (Sigma) in 1% (v/v) glacial acetic acid) to confirm the amount of protein transferred.

The membrane blotted with the anti-ERK1/ERK2 antibody was blocked at 4 °C overnight in 2% (w/v) skimmed dried milk and 0.05% (v/v) Tween-20 in PBS. Then it was incubated with anti-ERK1/ERK2 polyclonal antibody diluted 1:200 in blocking solution for 7 h at 4 °C and overnight at room temperature under agitation. Following three washes in 0.2% (w/v) non-fat dried milk and 0.5% (v/v) Tween-20 in PBS, the blot was incubated for two hours with alkaline phosphatase-conjugated anti-rabbit antibody (Sigma) diluted 1 : 1000 in blocking solution. The complexes were revealed by treatment with a nitro blue tetrazolium (NBT) and bromo-chloroindolyl-phosphate (BCIP) solution.

For the immunoblot with the anti-phospho MAPK antibody, the membrane was blocked at 4 °C overnight in 1% (v/v) blocking solution in TBS. The anti-phospho MAPK polyclonal antibody was used diluted 1 : 200 in 0.5% (v/v) blocking solution in TBS. Blots were incubated for 7 h at room temperature and overnight at 4 °C under agitation. Washes, secondary antibody incubation and detection procedures were performed using the BM Chemiluminescence Western Blotting Kit from ROCHE following the manufacturer's instructions. Each immunoblot was performed three times from independent experiments.

Results

In vitro culture stages during organogenic nodule formation

Four days after inoculation on morphogenesis-inducing medium, cambial cells of wounded-internodes start to divide (Fortes and Pais, 2000). These cambial cells kept dividing until prenodules were formed. At day 7, an incipient callus was formed at the wounded areas, and divisions can be observed in both cambial and cortical cells (see Fig. 4b). After 15 – 19 days in culture, cambial cells, which kept dividing tangentially, differentiated into vascular bundles. At this time, prenodular structures can be observed (see Fig. 4g). The prenodular structures kept increasing in size, giving rise to nodules 25 – 28 days after induction (Fortes and Pais, 2000). In nodules, both central vacuolated cells and peripheral meristematic cells can be noticed, as well as the vascular bundles (see Fig. 5a). Nodules were described by McCown and co-workers (1988) as dense independent cell clusters, which form a cohesive unit and display a consistent internal cell/tissue differentiation pattern and a high regenerative capacity. Nodules could undergo organogenesis resulting in several regenerated shoot buds at the periphery of nodular areas (see Fig. 5i).

Immunoblotting with antibodies to ERK1/2

The anti-ERK1/ERK2 is a polyclonal antibody that recognises in animals two polypeptides of 42 and 44 kDa. In tobacco, this antibody identified ERK1/2 homologues with 46 and 50 kDa (Cazale et al., 1999; Lebrun-Garcia et al., 1998). Using the anti-ERK1/2 antibody for immunoblots on *Humulus lupulus* protein extracts from different stages of nodule formation revealed two intense bands of approximately 46 and 50 kDa, which is the molecular weight reported for ERK1 and ERK2 in plants (Fig. 1). They were named HLERK1 and HLERK2.

During the process of nodule formation the amounts of HLERK1 and HLERK2 were different. In plants at time 0, the two HLERK proteins were detected at a very low level. At day 7, an increase of the HLERK2 isoform was observed and the HLERK1 maintained the basal level. At day 15, HLERK1 and HLERK2 maintained the levels from the previous stage. At the 28th and 45th days, an increase in the amount of HLERK1 was observed. HLERK1 and HLERK2 were equally present at this nodule developmental stage (Fig. 1). A third band visible in

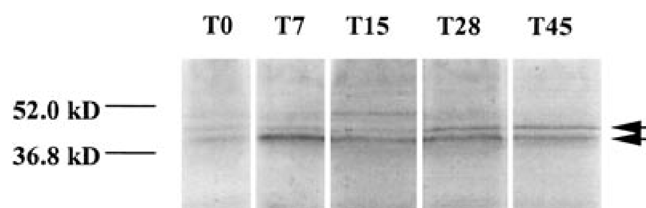


Fig. 1. Immunoblot of total protein extracts from different stages of the process of nodule induction and formation (time 0, 7, 15, 28 and 45 days) with anti-ERK1/2 antibody. Arrows indicate the 50- and 46-kDa ERK1 and ERK2 homologues, HLERK1 and HLERK2.

extracts at day 15 is probably unspecific, as it doesn't have the expected molecular weight described for MAPKs. In addition, this band does not appear in the immunoblot with the anti-phospho MAPK (see below).

Immunoblotting with antibodies to phospho-MAPKs

An immunoblot with the anti-phospho MAPK antibody revealed two bands corresponding to HLERK1 and HLERK2 (Fig. 2). During the process of nodule formation, differences were observed in the levels of active HLERK1 and HLERK2. Active HLERK proteins seem to be below the detection limit at time 0. At day 7, an increase of the active HLERK2 isoform was observed, whereas the active HLERK1 was not present. HLERK1 activity increased thereafter, from day 7 up to day 28. The HLERK2 isoform showed a further increase in the activity at day 15, then decreasing from day 15 up to 45 days. At 45th day, HLERK1 appeared to be more active than the HLERK2 isoform (Fig. 2).

Immunoblotting with ERK1/2 antibodies in induced internodes cultured with and without ROS inhibitor

Effects of NAC addition are better noticed in liquid medium, since the explant is entirely immersed in the medium. ROS inhibition at wounded areas was previously reported a few hours after culture initiation in liquid induction medium containing NAC (Fortes et al., 2002a). Therefore, the time point of 12 hours in induction medium was chosen to perform the immunoblotting with the anti-ERK1/2 antibody.

An immunoblot with the anti-ERK1/2 antibody on protein extracts from both treated and control internodes 12 h after induction, revealed the two expected polypeptides of 46 and 50 kDa, HLERK1 and 2 (Fig. 3). The internodes incubated on solid medium for 12 h, present the ERK2 homologue (HLERK2) more intense than the HLERK1. The third band present for this time is probably unspecific, not appearing on the other lanes. The control in liquid medium revealed low expression levels of HLERK2 isoform in the internodes whereas HLERK1 levels were similar to those from the internodes cultured on solid medium. ROS inhibition in liquid medium did not change the expression pattern of the HLERK1 and HLERK2 in the internodes, when compared to control internodes in liquid medium without NAC (Fig. 3).

Immunolocalization of HLERK1/HLERK2

Immunolocalization with anti-ERK1/ERK2 antibody on sections of internodes showed a weak signal in cortical cells at time zero (Fig. 4a). Autofluorescence was observed at the cutin layer surrounding epidermal cells and at vascular cells (Fig. 4a), both in sections incubated with anti-ERK1/2 and control sections with the buffer (data not shown).

At day 7, anti-ERK1/2 immunofluorescence signal was observed in the cytoplasm and nucleus of dividing cortical and cambial cells (Fig. 4d). Controls at day 7 without antibody revealed autofluorescence at the peripheral cutin layer and vascular cells (Fig. 4c).

Fifteen days after induction, prenodular cells showed a strong signal of HLERK1/2 proteins in the cytoplasm (Fig. 4h, k). ERK1/2 were also observed in the nucleus (Fig. 4h, k), as confirmed by nuclei DAPI staining (Fig. 4j, compare with Fig. 4k). At day 15, a control without antibody showed autofluorescence in peripheral cells surrounding the prenodule that accumulate cutin (Fortes et al., 2002b), and at the sclerenchyma (Fig. 4i).

At 28 days, HLERK1/2 were localized in the nodule, a more intense signal being observed in peripheral meristematic cells (Fig. 5b). In highly vacuolated cells of central nodular areas, HLERK1/2 appeared in the cytoplasm and nucleus (Fig. 5b′). The presence in the nucleus is confirmed by the co-localization of the signal with the DAPI staining (Fig. 5b″). In meristematic cells, HLERK1/2 were located in the cytoplasm and not in the nucleus (Fig. 5e, f). Sections of nodules treated without primary

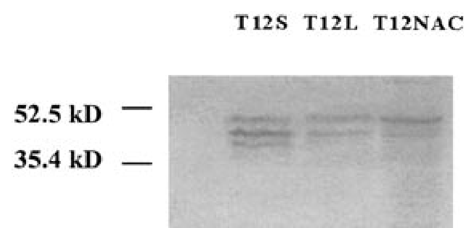


Fig. 3. Influence of culture conditions and ROS inhibitors on expression of HLERKs. Immunoblot of total protein extracts with anti-ERK1/ERK2 antibody. Internodes after 12 h on solid induction medium (T12S); wounded internodes after 12 h in liquid induction medium without (T12L) and with NAC (T12NAC).

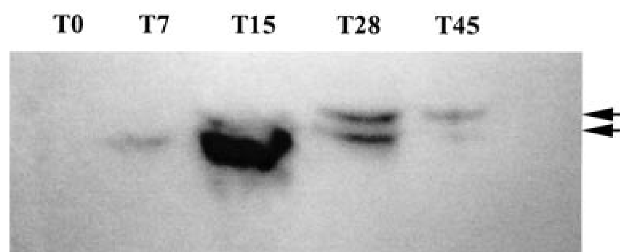
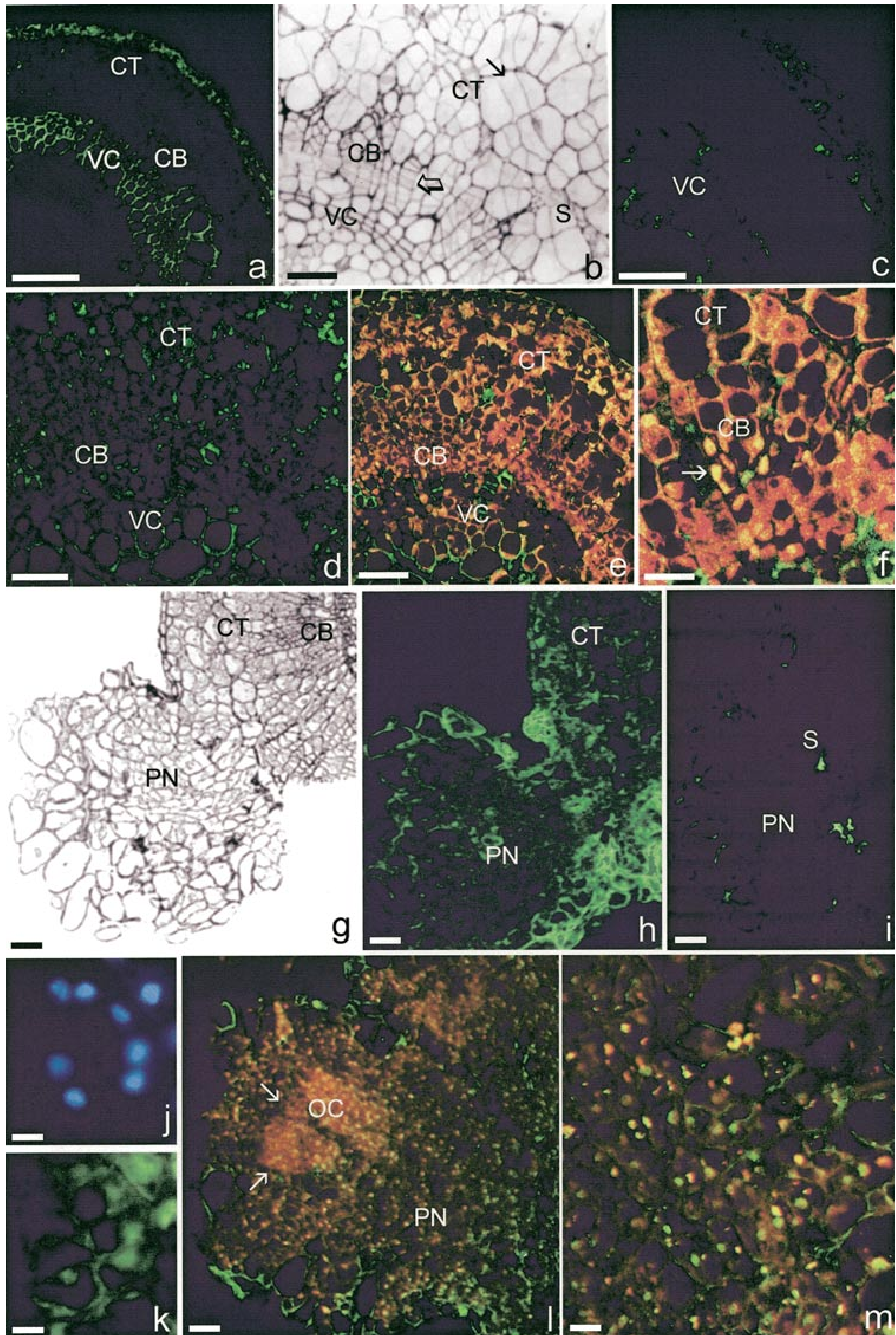


Fig. 2. Western blot of total protein extracts from different stages of the process of nodule induction and formation (time 0, 7, 15, 28 and 45 days) with anti-phospho MAPK antibody. Arrows indicate the activated HLERK1 and HLERK2.

Fig. 4. Localization of HLERK1/2 (a, d–f, h, k–m) and activated MAPKs (e, f, l, m) in internode sections at the time of wounding (a) and after 7 (a–f) and 15 days (g–m) in culture. Semi-thin sections of an internode region at day 7 (b) showing divisions at both cortical (thin arrow) and cambial cells (thick arrow) and of a prenodule at day 15 (g). Controls (omission of first antibody) are shown in (c) and (i). At time zero the HLERK1/2 signal was very weak in cortical cells, and autofluorescence was detected at epidermal cells, at vascular cells, and chloroplasts of subepidermal cells (a). At day 7 the fluorescent signal was observed in the cytoplasm and nucleus of dividing cortical and cambial cells (d) and co-localization (arrow) with activated MAPKs was observed (e, higher magnification in f). At day 15 the fluorescent signal was also observed in the cytoplasm and nucleus of prenodular cells (h, k) as revealed by magnification of a semi-thin section of a prenodule showing the nuclei from dividing prenodule cells labelled with DAPI (j; corresponding to k). Co-localization with activated MAPKs was observed in the cytoplasm and nucleus of prenodular cells (l, higher magnification in m), mostly in organising centers (arrows). CB – cambial cells; CT – cortical cells; OC – organizing center; PN – prenodule; S – sclerenchyma; VC – vascular cells. Bars in (a–e, g–i, l) = 100 μm; in (m) = 50 μm; in (f, j, k) = 25 μm. ▶


CT
VC
CB
a
CT
CB
VC
S
b
VC
c
CT
CB
VC
d
CT
CB
VC
e
CT
CB
f
CT
CB
PN
g
CT
PN
h
S
PN
i
j
k
OC
PN
l
m

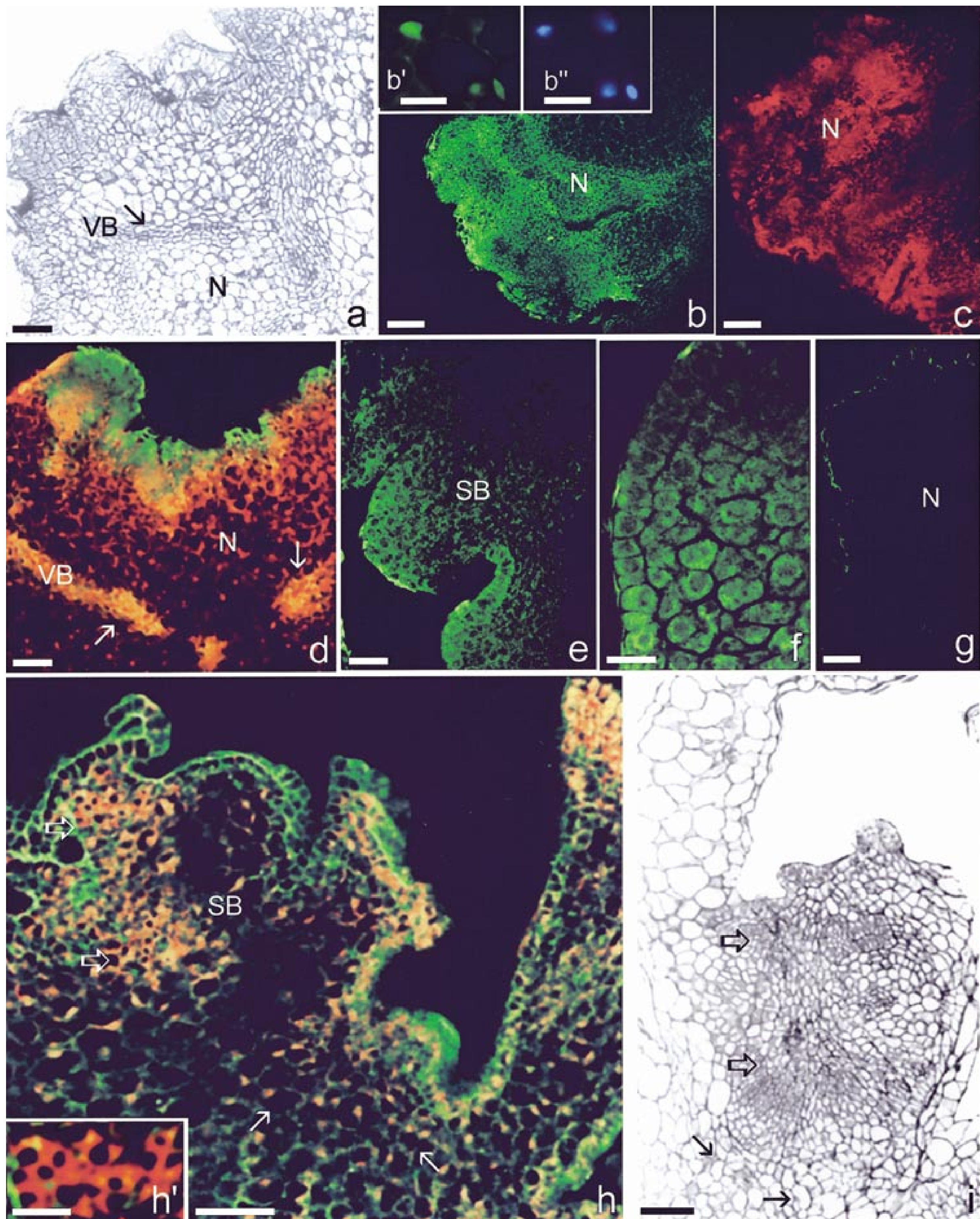


Fig. 5. Localization of HLERK1/2 (b, d, e, f, h) and activated MAPKs (c, d, h) in nodule sections at the time of 28 (a–c) and 45 days (d–i) in culture. Semi-thin sections of a nodule at day 28 (a) showing meristematic cells at the periphery, vacuolated cells in the inner regions of the nodule, and vascular bundles, and of a nodule at day 45 (i) showing a meristem (thick arrows) and leaf primordia. Highly vacuolated cells are indicated with thin arrows. A control (omission of first antibody) is shown in (g). At day 28 an intense fluorescent signal is observed in meristematic regions (b), appearing also in the cytoplasm and nucleus of vacuolated cells (b′; corresponding DAPI-stained cells shown in b″). An intense fluorescent signal with anti-phospho MAPK is observed in meristematic cells, which decreases towards the nodule centre (c). At day 45 anti-ERK1/2 and anti-phospho MAPK signals are observed in the cytoplasm and in the nucleus of peripheral cells and also in nodular organizing centers (arrows) in a section close to the nodule periphery (d). In a shoot bud the anti-ERK1/2 signal is observed in the cytoplasm of meristematic cells and not in the nucleus (e, higher magnification in f). In highly vacuolated cells of a peripheral nodule with meristematic areas and leaf primordia, active MAPKs co-localized with HLERKs in the cytoplasm and the nucleus (h, thin arrows). In meristematic cells, co-localization was only observed in the cytoplasm, and no signal was detected in the nucleus (thick arrows, and higher magnification shown in h′). N – nodule; VB – vascular bundles; SB – shoot bud. Bars in (a, d, e, h, i) = 100 μm; in (b, c, g) = 200 μ; in (b′, b″) = 50 μm; in (h′) = 25 μm.

antibody (control) showed autofluorescence only at the peripheral cutin layer (Fig. 5g).

Localization of activated HLERK1/2

The double immunolocalization with anti-phospho MAPK and anti-ERK1/2 antibody enabled the detection of activated HLERK1/2 in the internodes and nodules. Immunolocalization with anti-phospho MAPK antibody on sections of internodes at time zero showed a very week signal in cortical and cambial cells (data not shown).

At day 7, HLERK1/2 co-localized with the active phosphorylated MAPKs in the cytoplasm and the nucleus of cambial cells (Fig. 4e, f). The cambial cells are actively proliferating at this stage (Fortes and Pais, 2000). At day 15, HLERK1/2 and activated MAPKs co-localize in prenodular centers, mostly in the nucleus of prenodular cells (Fig. 4l, m). At day 28, activated MAPKs are detected in peripheral cells of the nodule, and the signal decreased from the periphery towards the nodule centre (Fig. 5c). In highly vacuolated cells of the nodule, activated MAPKs were observed in the nucleus and cytoplasm (Fig. 5d). Activated MAPKs co-localized with HLERK1/2 at the nodule periphery, in proliferating areas that will originate shoot meristem, and at vascular bundles (Fig. 5d; see Fig. 5a for histological identification). In shoot buds the anti-ERK1/2 signal is observed in the cytoplasm of meristematic cells and not in the nucleus (e, higher magnification in f). In highly vacuolated cells of peripheral nodules with meristematic areas and leaf primordia, active MAPKs co-localized with HLERKs in the cytoplasm and the nucleus (h, thin arrows). In meristematic cells, co-localization was only observed in the cytoplasm, and no signal was detected in the nucleus (thick arrows, and higher magnification shown in h′).

Discussion

Immunolocalization studies showed that organogenic nodule formation in *Humulus lupulus* var. Nugget involves the activation of MAPK homologues belonging to the ERK cascade. These MAPKs have been associated to cell proliferation and differentiation in animal cells (Marshall, 1995), and were also known to be activated by cytokines and oxidative stress induced by hydrogen peroxide (Buder-Hoffmann et al., 2001).

In hop cells, immunofluorescence studies carried out on sections of explants at time zero showed very low levels of HLERK1/2, as confirmed by Western blotting. The wound applied to internodes to induce nodule formation activates the production of reactive oxygen species (ROS) around wounded areas, which are still present in prenodules and nearly absent in nodules (Fortes and Pais, 1999). N-acetyl-L-cysteine (NAC), a precursor of glutathione, has been shown to inhibit ROS, by scavenging several types of free radicals. According to Fortes et al. (2002a) a concentration of 0.4 mM NAC is the maximal concentration that enables internodes to survive. Concentrations above this value led to cell death, and below 0.4 mM didn't promote complete ROS inhibition. ROS accumulation was detected using nitroblue tetrazolium (NBT) and 3,3′-diaminobenzidine (DAB) (Fortes et al., 2002a). Both ERK isoform homologues were recognised by the antibody in internodes cultured for 12 h on solid medium without inhibitor. A decrease in HLERK2 isoform was observed in the internodes cultured in liquid medium either in presence or absence of ROS inhibitor. This result suggests that the lower availability of oxygen in liquid medium and thus, lower production of ROS, is sufficient to inhibit HLERK2 expression. This way, it is not surprising that levels of HLERK2 are lower in explants cultured in liquid medium both in the presence or absence of ROS inhibitor. Inhibition of ROS resulted in a decrease of HLERK2 level, suggesting that this protein is induced by the production of ROS as a result of the wounding applied to the internodes. On the contrary, HLERK1 seems to be independent of ROS production. The fact that HLERK2 decreases in explants cultured in liquid medium, and since organogenic nodule formation was never achieved from induced internodes cultured in liquid medium, seems to indicate that HLERK2 synthesis may be activated by ROS formed in the sequence of wounding, accounting for a crucial role of this protein on organogenic nodule induction. However, additional studies are required to address ROS involvement in the hop ERK1/2 pathway. In animals, the levels of active forms of both ERK1 and ERK2 increase by exposing hippocampus to ROS donors, this activation being inhibited by pretreatment with NAC (Kanterewicz et al., 1998).

During the first stages (4–7 days after induction) cambial and cortical cells perceive the stimulus and start dividing, and after 15–19 days give rise to the prenodule (Fortes and Pais, 2000). At these stages, HLERK2 was detected in the cytoplasm of cortical and cambial cells, co-localizing with activated MAPKs. Immunofluorescence studies in animal (Brunet et al., 1999; Chen et al., 1992; Sanghera et al., 1992) and plant cells (Coronado et al., 2002; Prestamo et al., 1999) revealed the presence of MAPKs in the cytoplasm and nucleus. In hop, these proteins were localized in the nucleus since day 7 after induction until nodule and meristem formation. During cell differentiation in prenodules active HLERKs were localized, mostly in the nucleus of dividing cells and organizing centers, suggesting that these active MAPKs are translocated to the nucleus where they probably activate nuclear targets. In animals, the regulation of the MAPK accessibility to the nucleus is a key signalling event by which cells may control the intensity and temporal activation of genes during cell growth and differentiation (Brunet et al., 1999). The presence of HLERKs in the nucleus of prenodular cells may be related to the initiation of cell differentiation into nodules. Nuclear presence of plant ERK1/2 homologues during pollen maturation (a differentiation process) has been recently reported (Coronado et al., 2002). HLERKs are highly present in meristematic dividing cells at the periphery of nodules, mostly in the cytoplasm. At early pollen embryogenesis, when the cell cycle is activated and cells start proliferating, ERK1/2 homologues have been immunolocalized both in the cytoplasm and the nucleus (Coronado et al., 2002). The results reported in the present paper suggest that in dividing cells of the hop organogenic nodules, HLERKs are localized either in the nucleus or in the cytoplasm at different morphogenic stages. Similar results have been reported for different mammalian cell types entering differentiation and proliferation processes (Marshall, 1995). In mammalian PC12 cells (a tumour cell line derived from the rat adrenal medulla), sustained activation and subsequent nuclear localization of ERK leads to differentiation, whereas in fibroblasts, sustained activation and nuclear localization is associated with proliferation (Marshall, 1995). The cytoplasmic localization of HLERKs found in dividing nodular cells provides evidence that different plant cell

types and processes could lead to different localization patterns, as also found in mammals (Marshall, 1995), and also determine the activation of these proteins. Although present to a less extent in vacuolated non-dividing cells, HLERKs are found in the cytoplasm and nucleus. Activated HLERKs can be also observed in the nucleus of nodular cells that are differentiating into shoots. This duration and/or magnitude of ERK activation is critical for cell signalling, as reported for animals (Marshall, 1995), being in part responsible for different signalling responses through the same kinase. Cells enter proliferation or differentiation by using either transient or sustained activation of ERKs, this being related to its entrance or not in the nucleus (Cowley et al., 1994; Mansour et al., 1994; Marshall, 1995).

Our results suggest that along the process of nodule formation, HLERK1 and HLERK2 could respond to diverse stimuli activating different intracellular responses, either cell proliferation or cell differentiation. In hop, HLERK2 seems to act throughout the organogenic process, whereas HLERK1 may be involved in stages when daughter nodules and meristem formation occur (28 and 45 days after induction).

Although morphogenic processes such as somatic embryogenesis and organogenic nodule formation play pivotal roles in plant biotechnology, little is known about the signals involved in their induction and development. Studies on involvement of ERK1/2 and active MAPK signaling in proliferation and differentiation have already been performed in animals (Cowley et al., 1994; Mansour et al., 1994; Marshall, 1995). In plants this pathway has been reported to be involved in different proliferation and developmental processes (Bögre et al., 2000; Coronado et al., 2002; Hirt, 2000; Huang et al., 2002; Prestamo et al., 1999). Up to our knowledge, this is the first report mentioning the differential expression and activation of two ERK isoform homologues throughout a plant morphogenic process.

Acknowledgements. The authors thank Mrs M. A. Ollacarizqueta (CIB, CSIC, Spain) for excellent technical assistance in confocal microscopy. The authors also thank J. C. Costa for help with ROS inhibition experiments. Funding for this work was provided by the Portuguese Foundation for Science and Technology through a PhD grant to M. S. Silva (PRAXIS XXI /BD/13730/97). This work was also supported by the Portuguese Project PCTI/1999/BME/32734, by the Spanish Projects DGESIC PB 98–0678, MCyT B052002–03572 and CAM 076/0054/2000, and by the Portuguese-Spanish Joint Project Acción Integrada HP2000–0065.

References

Batista, D., Sousa, M. J., Pais, M. S., 1996. Plant regeneration from stem and petiole-derived callus of *Humulus lupulus* L (Hop) clone Bragança and var Brewer's Gold. In Vitro Cell Dev. Plants 32, 37–41.

Batista, D., Ascensao, L., Sousa, M. J., Pais, M. S., 2000. Adventitious shoot mass production of hop (*Humulus lupulus* L.) var. Eroica in liquid medium from organogenic nodule cultures. Plant Sci. 151, 47–57.

Bögre, L., Calderini, O., Binarova, P., Mattauch, M., Till, S., Kiegerl, S., Jonak, C., Pollaschek, C., Barker, P., Huskisson, N. S., Hirt, H., Heberle-Bors, E., 1999. A MAP kinase is activated late in plant mitosis and becomes localized to the plane of cell division. Plant Cell 11, 101–113.

Bögre, L., Meskiene, I., Heberle-Bors, E., Hirt, H., 2000. Stressing the role of MAP kinases in mitogenic stimulation. Plant Mol. Biol. 43, 705–718.

Bradford, M. M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Brunet, A., 1998. Signal transduction from the membrane to the nucleus. variations on common themes. Bull. Cancer 85, 527–537.

Brunet, A., Roux, D., Lenormand, P., Dowd, S., Keyse, S., Pouyssegur, J., 1999. Nuclear translocation of p42/p44 mitogen-activated protein kinase is required for growth factor-induced gene expression and cell cycle entry. EMBO J. 18, 664–674.

Buder-Hoffmann, S., Palmer, C., Vacek, P., Taatjes, D., Mossman, B., 2001. Different accumulation of activated extracellular signal-regulated kinases (ERK 1/2) and role in cell-cycle alterations by epidermal growth factor, hydrogen peroxide, or asbestos in pulmonary epithelial cells. Am. J. Respir. Cell Mol. Biol. 24, 405–413.

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

Cazale, A. C., Droillard, M. J., Wilson, C., Heberle-Bors, E., Barbier-Brygoo, H., Lauriere, C., 1999. MAP kinase activation by hypoosmotic stress of tobacco cell suspensions. towards the oxidative burst response? Plant J. 19, 297–307.

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

Coronado, M. J., Gonzalez-Melendi, P., Segui, J. M., Ramirez, C., Barany, I., Testillano, P. S., Risueno, M. C., 2002. MAPKs entry into the nucleus at specific interchromatin domains in plant differentiation and proliferation processes. J. Struct. Biol. 140, 200–213.

Cowley, S., Paterson, H., Kemp, P., Marshall, C. J., 1994. Activation of MAP kinase kinase is necessary and sufficient for PC12 differentiation and for transformation of NIH 3T3 cells. Cell 77, 841–852.

Fabregat, I., Herrera, B., Fernández, M., Álvarez, A. M., Sánchez, A., Roncero, C., Ventura, J.-J., Valverde, A. M., Benito, M., 2000. Epidermal growth factor impairs the cytochrome C/caspase-3 apoptotic pathway induced by transforming growth factor beta in rat fetal hepatocytes via a phosphoinositide 3-kinase-dependent pathway. Hepatology 32, 528–535.

Fortes, A. M., Pais, M. S., 1999. Elemental changes, reactive oxygen species and lipoxygenase activity during induction of organogenesis from internode derived nodules of *Humulus lupulus* var. Nugget. Abstract. Plant cell biology and biotechnological applications research conference (European Science Foundation Conference) Rolduc, The Netherlands, p. 40.

Fortes, A. M., Pais, M. S., 2000. Organogenesis from internode-derived nodules of *Humulus lupulus* var. Nugget (Cannabinaceae). Histological studies and changes in the starch content. Am. J. Bot. 87, 971–979.

Fortes, A. M., Costa, J. C., Testillano, P., Risueno, M. C., Pais, M. S., 2002a. Reactive oxygen species and expression of LOX during morphogenesis in hop. Abstract. Thiols and Redox Regulation – NATO Advanced Research Workshop, Pisa, Italy.

Fortes, A. M., Testillano, P. S., Risueno, M. C., Pais, M. S., 2002b. Studies on callose and cutin during the expression of competence and determination for organogenic nodule formation from internodes of *Humulus lupulus* var. Nugget. Physiol. Plant. 116, 113–120.

Hirt, H., 2000. Connecting oxidative stress, auxin, and cell cycle regulation through a plant mitogen-activated protein kinase pathway. Proc. Natl. Acad. Sci. USA 97, 2405–2407.

Huang, H. J., Fu, S. F., Tai, Y. H., Chou, W. C., Huang, D. D., 2002. Expression of *Oryza sativa* MAP kinase gene is developmentally regulated and stress-responsive. Physiol. Plant. 114, 572–580.

Kanterewicz, B. I., Knapp, L. T., Klann, E., 1998. Stimulation of p42 and p44 mitogen-activated protein kinases by reactive oxygen species and nitric oxide in hippocampus. J. Neurochem. 70, 1009–1016.

Lebrun-Garcia, A., Ouaked, F., Chiltz, A., Pugin, A., 1998. Activation of MAPK homologues by elicitors in tobacco cells. Plant J. 15, 773–781.

Leon, J., Rojo, E., Sanchez-Serrano, J. J., 2001. Wound signalling in plants. J. Exp. Bot. 52, 1–9.

Ligterink, W., 2000. MAP kinases in plant signal transduction. How many, and what for?, in: Hirt, H. (Ed.), Results and Problems in Cell

Differentiation, MAP Kinases in Plant Signal Transduction. Springer-Verlag, Berlin, Heidelberg, Germany, Vol. 27, pp. 11–27.

Mansour, S. J., Matten, W. T., Hermann, A. S., Candia, J. M., Rong, S., Fukasawa, K., Vande Woude, G. F., Ahn, N. G., 1994. Transformation of mammalian cells by constitutively active MAP kinase kinase. Science 265, 966–970.

Marshall, C. J., 1995. Specificity of receptor tyrosine kinase signaling. transient versus sustained extracellular signal-regulated kinase activation. Cell 80, 179–185.

McCown, B. H., Zeidin, E. L., Pinkalla, A. H., Dedolph, R. R., 1988. Nodule culture. A developmental pathway with high potential for regeneration, automated micropropagation, and plant metabolite production from woody plants, in: Hanover, J. W., Keathley, D. E. (Eds.), Genetic Manipulation of Woody Plants. Plenum Press, New York, pp. 146–166.

Meskiene, I., Hirt, H., 2000. MAP kinase pathways. Molecular plug-and-play chips for the cell. Plant Mol. Biol. 42, 791–806.

Murashige, T., Skoog, F., 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol. Plant. 15, 473–497.

Prestamo, G., Testillano, P. S., Vicente, O., Gonzalez-Melendi, P., Coronado, M. J., Wilson, C., Heberle-Bors, E., Risueno, M. C., 1999. Ultrastructural distribution of a MAP kinase and transcripts in quiescent and cycling plant cells and pollen grains. J. Cell Sci. 112, 1065–1076.

Rentrop, M., Knapp, B., Winter, H., Schweizer, J., 1986. Aminoalkylsilane-treated glass slides as support for in situ hybridization of keratin cDNAs to frozen tissue sections under varying fixation and pretreatment conditions. Histochem. J. 18, 271–276.

Sanghera, J. S., Peter, M., Nigg, E. A., Pelech, S. L., 1992. Immunological characterization of avian MAP kinases. evidence for nuclear localization. Mol. Biol. Cell 3, 775–787.

Schenk, P. W., Snaar-Jagalska, B. E., 1999. Signal perception and transduction. the role of protein kinases. Biochim. Biophys. Acta 1449, 1–24.

Seo, S., Ohashi, Y., 2000. Mitogen-Activated Protein Kinases and Wound Stress, in: Hirt, H. (Ed.), Results and Problems in Cell Differentiation. MAP Kinases in Plant Signal Transduction. Springer-Verlag, Berlin, Heidelberg, Germany, Vol. 27, pp. 53–60.

Shapiro, P. S., Vaisberg, E., Hunt, A. J., Tolwinski, N. S., Whalen, A. M., McIntosh, J. R., Ahn, N. G., 1998. Activation of the MKK/ERK pathway during somatic cell mitosis. direct interactions of active ERK with kinetochores and regulation of the mitotic 3F3/2 phosphoantigen. J. Cell Biol. 142, 1533–1545.

Stone, J. M., Walker, J. C., 1995. Plant protein-kinase families and signal-transduction. Plant Physiol. 108, 451–457.

Takabe, T., Takabe, T., Akazawa, T., 1986. Biosynthesis of P700-chlorophyll-a protein complex, plastocyanin, and cytochrome B6/F complex. Plant Physiol. 81, 60–66.

Testillano, P. S., Gonzalez-Melendi, P., Ahmadian, P., Fadon, B., Risueno, M. C., 1995. The immunolocalization of nuclear antigens during the pollen developmental program and the induction of pollen embryogenesis. Exp. Cell Res. 221, 41–54.