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Research Article

Alfalfa Mob1-like proteins are involved in cell proliferation and are localized in the cell division plane during cytokinesis

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ABSTRACT

Mps-one-binder (Mob) proteins play a crucial role in yeast cytokinesis. After cloning two *Mob1*-like genes, *MsMob1-A* and *MsMob1-B* from alfalfa (*Medicago sativa* L.) we show that, although they are constitutively expressed in roots, stems, leaves, flowers and pods, their transcripts and proteins are mostly produced in actively proliferating tissues. A polyclonal antibody specifically raised against MsMob1 proteins was used for immunolocalization studies in synchronized root tip cells. The subcellular localization of MsMob1-like proteins is demonstrated to be cell cycle-regulated. Cytoplasmic localization is faint and diffused during G_1 and S. It becomes concentrated in punctuate and fibrillar structures in G_2 as well as M phase. At the stage of cytokinesis, the protein is found at the emerging cell plate marking the progressive formation of the septum. Mob1 proteins partially co-localize with microtubules structures functionally related to the spindles and important for cytokinesis in eukaryotic cells. The *MsMob1* expression cannot rescue the lethality of the yeast *mob1* mutant, suggesting that interaction of Mob1 proteins with their effectors may be species-specific. Localization of Mob1 proteins in the inner layer of the root cap indicates an additional function for this class of proteins in plants, which is likely related to the onset of programmed cell death.

Introduction

Cytokinesis is a key point in cell proliferation and fundamental for the growth and development of all eukaryotic organisms, including higher plants. Although cytokinesis can take place with different modalities in yeasts, animals and plants, the basic control mechanisms underlying this process are remarkably conserved during evolution. For example, many eukaryotic cells use a contractile actomyosin ring that creates a cleavage furrow which partitions the cell into two lobes. In plants, spatial control of cell division mainly depends on specific cytoskeletal structures such as the pre-prophase band and the phragmoplast [1,2]. Generally, during interphase microtubules radiate from the nucleus to the cell membrane

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and span the cell cortex in an orderly manner. Then, microtubules from the centrally positioned nucleus accumulate transversely at the shortest distance from the cortex [3,4]. Both the tubulin and actin accumulate in a medial ring on the plasma membrane to form the pre-prophase band which narrows in early mitosis to mark the future division plate. The onset of cell division is catalyzed by creation of the phragmoplast, a structure composed of microtubules, actin filaments and vesicles, which is thought to develop from the mitotic spindle remnants and required to lay down the new cell wall during cytokinesis. How the phragmoplast is assembled and exactly how it promotes cytokinesis is still not clearly understood.

Several genes playing a role in plant cytokinesis have been discovered, mainly through the characterization of mutants that display either defects in establishing a division plane or aberrant formation of the cell plate. Several *Arabidopsis* genes involved in cytokinesis have been cloned. Of these, some have been implicated in polarized vesicle trafficking (e.g., *SCD1*), in vesicle fusion to form the cell plate (*KNOLLE* and *KEULE*), in primary cell wall biosynthesis (*KORRIGAN* and *CYT1*), as well as phragmoplast-mediated expansion and cell plate guidance (*HINKEL*) [5–11]. Growing evidence indicates that protein phosphorylation is crucial in regulating plant cytokinesis. In particular, the mitogen-activated protein kinase (MAPK) cascade promotes cell plate formation and reorganization of microtubules for phragmoplast expansion [12]. Given the complexity of the process, other plant cytokinetic machinery components need to be identified.

Recently, a molecular dissection of plant cytokinesis and phragmoplast structure was carried out by Van Damme et al. [13]. The *Arabidopsis* genome was searched for cytoskeleton and cytokinesis-related genes. Several kinases, phosphatases and putative mitotic exit network (MEN)/septation initiation network (SIN) components were found in the nucleus, while actin- and microtubule-binding proteins, regulatory proteins and putative cell cycle-controlled proteins were associated with separate sub-regions of the phragmoplast and/or the cell plate showing the existence of distinct structural components of the cytokinetic apparatus.

A family of structurally related proteins (Mob, Mps-one-binder), which includes highly conserved cell cycle-regulated proteins that likely function as protein kinase activating subunits, has been demonstrated to be important for both mitosis completion and cytokinesis in budding yeast [14]. Its founding member, Mob1, was identified in *Saccharomyces cerevisiae*, where it is an essential component of the MEN, a complex signal transduction cascade that co-ordinates mitotic exit with cytokinesis [15]. This pathway promotes the inactivation of the mitotic Cdk1-cyclin B complexes and drives mitotic exit by leading to the release from the nucleolus and subsequent activation of the Cdc14p phosphatase [15,16]. Although inactivation of Cdk1–cyclin B complexes is required for cytokinesis, the MEN is shown to be essential for cytokinesis, and in particular for actomyosin ring contraction and septum deposition also independently of its role in mitotic exit. In fact, when MEN function is abrogated in conditions where mitotic exit is through artificial suppression of mitotic CDK activity, cytokinesis does not take place [17–19]. Remarkably, the fission yeast septation initiation pathway (SIN) is organized similarly to the MEN but is involved only in cytokinesis and not in mitotic exit (reviewed in [20]). Components of the MEN/SIN localize on both spindle pole bodies and the division septum in both budding and fission yeast (reviewed by [16,20]). Mob1 has been proposed to function as an activating subunit for the MEN/SIN Dbf2/Sid2 protein kinase, which is in turn required for the release of Cdc14p from the nucleolus in late mitosis [21,22]. A second member of the MOB family, the Mob2 protein, associates and activates a Dbf2-related protein kinase (Cbk1p) that regulates daughter cell-specific gene expression and actin cytoskeletal polarity [23,24].

Mob1-related genes have been found in animal [25–27] and plant cells [13,28,29]. The study of a spontaneous lethal mutation in a *Drosophila* Mob1 gene has recently implicated the Mob protein family in the control of animal cell proliferation and apoptosis [30]. Moreover, the identification of the animal Dbf2 homologous proteins NDR (Nuclear Dbf2-Related) interacting with Mob1-related proteins, and the determination of the human and *Xenopus laevis* Mob protein 3D structures, may mean that Mob proteins act as kinase activating subunits even in higher eukaryotes.

Plant genomes also contain Mob1-related genes. Members belonging to the MOB gene family from alfalfa (*Medicago sativa* L.) were recently isolated and studied in reproductive organs by Citterio et al. [29]. An alfalfa Mob1-like gene was shown to be specifically expressed during meiosis and gametogenesis. In particular, Mob1 gene products were found in degenerating megaspores of ovules and tapetum cells of anthers undergoing programmed cell death, which means that, as in the *Drosophila*, these proteins may be involved in apoptosis. A Mob1 gene from *Arabidopsis* has also been thought to be involved in plant cytokinesis [13].

We now report the characterization of the alfalfa Mob1-like genes (*MsMob1-A* and *MsMob1-B*), previously isolated by our group. Polyclonal antibodies specifically raised against alfalfa Mob1 proteins recognized different isoforms of at least two distinct proteins which are likely products of the two genes. The subcellular localization of these proteins is consistent with a role of Mob1-like proteins in plant cell proliferation and cytokinesis. Nevertheless, despite the high similarity between *M. sativa* Mob1 and *S. cerevisiae* Mob1 primary sequences, expression of alfalfa *Mob1* cannot rescue the lethality of yeast *mob1* mutant cells at the restrictive temperature, suggesting that interaction of Mob1 proteins with their effectors may be species-specific.

Materials and methods

Plant material

Medicago sativa subsp. *coerulea* (Less.) Schm. (2*n* = 2x = 16) clone was used to isolate Mob1-like genes and to assess their organization and expression in roots, stems, leaves, flowers and pods in replicated experiments. Alfalfa root tip cells were also studied through in situ hybridization and immunocytochemical assays.

Sequence data analysis

Mob1-like gene homologues were searched in public databases with BLASTN and BLASTX applications [31] to compare nucleotide and translated sequences. The most significant amino acid sequences of plant and other eukaryotic organisms were selected after multiple sequence alignments. The resulting substitution matrices were adopted to construct dendrograms with a CLUSTALW program [32]. A pair-wise comparison between the consensus sequence obtained from Mob1-like plant proteins and the MOB1/phocein domain (pfam03637) was also performed.

Temporal gene expression analysis by RT-PCR

RT-PCR experiments were performed to study expression patterns of alfalfa Mob1-like genes *MsMob1-A* and *MsMob1-B* using primers designed on discriminating sites of their ORF sequences. The 5′ to 3′ sequences for the principal oligonucleotide primers used for analyzing the mRNA expression levels of the alfalfa Mob1-like genes are as follows: $pMob1_{For67}$: 5′ GGAAGTAAGGGTGCTCAACTTCAAAAACAC 3′; $pMob1_{For201}$: 5′ TCAAGTGAATACCATGTTTGGTACGTTGAC 3′; $pMob1_{For303}$: 5′ CGTTACTATCAAGAAACCAATAGAGGTA 3′; $pMob1_{Rev399}$: 5′ AAAATTGTTTCATCATCTAGCTGAGATTCC 3′ (specific for *Mob1-B*); $pMob1_{Rev515}$: 5′ ACAATCTTCTGAAAATGTGAGTGATAAAC 3′ (specific for *Mob1-A*); $pMob1_{Rev533}$: 5′ GCTTCTTCCT TCAAACTCACAATCTTCT 3′ (the number indicates the sequence length separating the primer 5′ end nucleotide from the start codon of the shared MOB1 domain).

For cDNA synthesis, a GeneAmp EZ *rTth* PCR kit from Applied Biosystems was used according to the manufacturer's instructions. The cDNA was eluted ten times and used as template for PCR analysis with primers specific for the Mob1-like gene. The reaction mixture of 50 μl contained 10 pmol of each primer, 1× PCR buffer (50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris–HCl), 1 mM of dNTPs and 1 U of *Taq* DNA polymerase (Pharmacia Biotech). The following cycling conditions ensured optimal primer selectivity: after a first step of 3 min at 94°C, 30 to 36 cycles of 94°C for 40 s, 55°C for 50 s, and 72°C for 1 min were performed. In preliminary experiments, PCR products were collected after 24, 26, 28, 30, 32, 34 and 36 cycles then assayed in 2% agarose gels to determine the amplification linearity.

The two Mob1-like gene transcripts were assayed in roots, stems, leaves, flowers and pods. Moreover, RT-PCR analysis was also carried out in root tips at 12 and 48 h from the beginning of seed imbibition, and in old (fully differentiated) and young (not differentiated) leaves to elucidate the involvement of the Mob1-like gene in cell cycle. Replicated experiments were performed using different ORF-specific primer combinations. Expression of the actin gene was used as constitutive control in all experiments.

The corrected gross counts (absolute gross counts subtracted by the background levels from corresponding lanes) for each PCR product were quantified using Kodak 1D image analysis software. To evaluate the relative amount of mRNA for each tissue and stage, the corrected gross counts for the target gene were normalized against those of the housekeeping actin mRNA. Values were expressed in gross count arbitrary units and standard deviations calculated for replicated experiments.

In situ hybridization

Alfalfa root tips from germinated seeds were fixed with 4% paraformaldehyde, 0.2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 16 h at 4°C and embedded in Paraplast Plus (Sigma-Aldrich). Sections of 7–10 μm were cut using a microtome (RM 2135 Leica, Germany) and collected in xylane-coated slides. The slides were de-paraffinized, treated with 5 μg/ml proteinase K and hybridized with sense and antisense riboprobes in 50% formamide at 45°C overnight. A 400 bp long cDNA fragment of the alfalfa Mob1-like gene, previously tested for its sequence specificity [29], was cloned into the TOPO vector (Invitrogen) and transcribed in vitro to obtain DIG-UTP (Roche) labelled RNA sense and antisense probes using T7 and Sp6 polymerases. After hybridization the slides were washed in 2× SSC at 45°C then treated with 20 μg/ml RNaseA. DIG detection and signal visualization were carried out using NBT and BCIP (Roche). Images were acquired using a Leica DC 300F camera.

Immunochemical procedures

To produce an antibody for the detection of the alfalfa Mob1-like proteins, a peptide from the N-terminal region of the protein, LGSRNQKTFRPKKSA, was selected on the basis of the predicted protein structure and synthesized by Sigma Genosys. Considering all amino acid sequences present in protein databases, the peptide is specific to yeast, animal and plant Mob1 members and it is not present in Mob2 proteins. At the C-terminus, the peptide was conjugated to keyhole limpet haemocyanin carrier protein with EDC chemistry. The coupled peptide was injected into two rabbits. The antiserums were affinity-purified on the peptide for immuno-blotting and -localization. Antibody specificity was preliminarily tested by immunoblotting with plant protein extracts.

Negative controls were performed by saturating the antibody with the correspondent peptide (1:1) at 4°C overnight. Antibody specificity was also established by in situ immunolocalization using either sectioned tissues or isolated cells. In all cases no fluorescence was detectable when the purified anti-Mob1 antibody was saturated with the correspondent peptide (1:1) at 4°C overnight.

Electrophoresis and Western blot analysis

Total protein extracts were obtained from alfalfa root tip and leaf at different development stages. 1 g of plant material was frozen in liquid nitrogen, transferred to a pre-chilled mortar, ground into a fine powder and homogenized in four volumes of extraction buffer with 60 mM Tris–HCl pH 6.8, 2% β-mercaptoethanol, 0.3% SDS and 1× protease inhibitor cocktail for plant (Sigma). The homogenate was centrifuged at 15,000 × *g* for 15 min at room temperature. The supernatant was boiled and subsequently precipitated by adding nine volumes of cold acetone and placing at –20°C for 30 min. After centrifugation at 15,000 × *g* for 10 min at 4°C, the pellet was

washed twice with 1 ml cold acetone and newly centrifuged. It was air-dried and resuspended in urea-thiourea solution (7 M urea, 2 M thiourea, 20 mM DTT). After centrifugation at 15,000 × *g* for 10 min at 4°C, the protein concentration in the supernatant was measured with the Bradford assay with a dye reagent from Bio-Rad (Hercules). Protein separation was carried out with single dimensional SDS-PAGE and two-dimensional gel electrophoresis.

Samples for 1D SDS-PAGE were mixed 1:1 (v:v) with 2× sample buffer (60 mM Tris–HCl pH 6.8, 1 mM β-mercaptoethanol, 20% glycerol, 3% SDS, 0.002% bromophenol blue); 30 μg of proteins were loaded on 12% gels and either stained with Coomassie Blue or transferred to nitrocellulose membranes (Amersham Bioscience). Moreover, 2D electrophoresis was performed using 200 μg of extracted proteins. For the first dimension, extract was applied to a 11 and 17 cm long immobilized pH gradient (IPG) strips, providing a non-linear pH 3 to 10 gradient (BioRad). Isoelectric focusing was performed in a Protean IEF cell (BioRad). After the first dimension separation, the IPG strips were subjected to SDS PAGE on 12.5% gels and either stained with Coomassie G-250 dye (GelCode Blue Stain Reagent, Pierce) or transferred to nitrocellulose membranes (Amersham Bioscience).

For Mob1 protein immunodetection, 1D and 2D membranes were incubated with 5% non-fat dry milk in TBS-T buffer (20 mM Tris–HCl pH 7.4, 150 mM NaCl, 0.1% Tween20) and then with 1 μg ml^{-1} anti-Mob1 primary antibody. After 1 h, they were washed in TBS-T and probed with horseradish peroxidase conjugated secondary antibody (Amersham Bioscience). Bound antibodies were visualized using chemiluminescent detection system ECL (Amersham Bioscience). Phosphatase treatment was performed on total protein extracts using Lambda Protein Phosphatase, λPPase (New England Biolabs Inc.) following the protocol described by Yamagata et al. [33].

Flow cytometry

To check the cell proliferation condition in the plant tissues used for immunoblotting and immunofluorescence microscopy, flow cytometry was applied following the protocols previously described by Citterio et al. [34]. Briefly, nuclear suspensions from root tips after 12 and 48 h from the beginning of seed imbibition, and from old and young leaves were analyzed with a Bryte-HS (Bio-Rad) flow cytometer equipped with a high pressure mercury-xenon lamp and with 365 ± 5 nm excitation, 390 nm dichroic mirror and 450–490 nm band pass filters.

Immunofluorescence microscopy

Alfalfa seeds were surface-sterilized with sodium hypochlorite (0.6% active chlorine) for 10 min, washed under running tap water for 15 min and left to imbibe water for 15 h. To obtain isolated meristematic cells synchronized in mitosis, the seeds were grown in Petri dishes with distilled water up to 60 h from the beginning of imbibition. Water was then replaced with 3 mM hydroxyurea, HU (Sigma). After 12 h (72 h from the start of imbibition), treated plantlets were washed thoroughly and allowed to recover in distilled water for about 10 h. Root tips (2 mm long) were then cut, fixed in 4% (v/v) paraformaldehyde (10% solution, methanol-free by Polysciences) in Tris buffer [10 mM Tris, 10 mM NaEDTA and 100 mM NaCl, pH 7.4] for 24 h at 4°C and washed thoroughly in the cold buffer for 30 min. After fixation, the root tips were carefully squashed between two slides to release individual cells and dried overnight. After rehydration in Tris buffer for 30 min, the cell walls were digested for 4 h with a solution of 4% cellulase R-10, 1% pectinase (Sigma), 3% macerozyme (Sigma), 0.05 M citric acid, 0.6 M mannitol and 0.1% triton, pH 4.8. The slides were then washed three times in Tris buffer and immersed in –20°C ethanol to further permeabilize the cells. After 10 min, the slides were washed three times in Tris buffer and processed for immunostaining. Before antibody incubation, non-specific reactivity was blocked by applying 500 μl 1% powder milk dissolved in Tris buffer, for 1 h at 25°C. The anti-Mob1 specific antibody was used at 5 μg/ml overnight at 4°C. After washing with Tris buffer, the slides were incubated with a 1:200 diluted goat anti-rabbit Ig G (L+H) Alexa Fluor-488 (Molecular Probe) for 30 min at room temperature and counterstained with 0,2 μg ml^{-1} DAPI in Tris. A negative control in which the anti-Mob antibody had been pre-adsorbed on the peptide used for immunization was included in each experiment. The slides were mounted in 95% glycerol in Tris (1:1) and examined using a Zeiss Axioplan microscope, equipped with standard fluorescence filter sets and connected to a video camera (Media Cybernetics).

Qualitative and quantitative image analyses were carried out using the Image-Pro plus program (Media Cybernetics). For double labeling, the microtubule staining was performed with the mouse monoclonal antibody DM1A (Sigma) diluted 1:50 and with the goat anti-mouse Ig G (L + H) Alexa fluor-594 (Molecular Probe) diluted 1:200, just after Mob1 detection. Alternatively the anti-α tubulin and the anti-Mob1 antibodies were mixed and simultaneously applied. The two secondary antibodies were also mixed and used to visualize the antigens. The two staining procedures produced identical results.

The same immunostaining protocols were also used for Mob1 detection in the semi-thin alfalfa root tip sections included in LR gold resin according to the manufacturer's instructions (Polysciences).

Plasmid constructions and yeast genetic manipulations

To construct the *SIC1*-bearing 2 μm plasmid (pSP339) a *Xba*I/*Eco*RI fragment containing a *SIC1* version tagged with four HA epitopes before the stop codon (a kind gift from E. Schwob) was cloned in *Xba*I/*Eco*RI of Yeplac112.

To clone *MsMob1-A* under the yeast *GAL1* promoter, an *Xba*I PCR product containing the *MsMob1-A* coding region was cloned in the *Xba*I site of a *GAL1*-bearing YIplac211 vector. Integration of the resulting pSP288 plasmid was directed to the *URA3* locus of isogenic wild type and *mob1-77* strains (kindly supplied by Francis Luca; [14]) by *Stu*I digestion. The resulting transformants (ySP5450 and ySP5451 for the wild type as well as ySP5452 and ySP5453 for the *mob1-77* mutant strain) were grown in YEP medium (1% yeast extract, 2% bactopeptone, 50 mg/l adenine) containing raffinose (2%, YEPR) and raffinose plus galactose (1%, YEPRG) to induce the *GAL1* promoter.

Protein extracts were made by TCA precipitation as previously described [35]. For the drop test, cells were grown

overnight in YEP medium containing raffinose (2%). After determination of cell culture concentrations, serial 1:5 dilutions were spotted on YEP medium containing either glucose (*GAL1* promoter off) or raffinose and galactose (*GAL1* promoter on).

Results

Genomic organization and bioinformatic characterization of the Mob1-like genes

Four distinct Mob1-like gene family members were recently cloned in alfalfa by Citterio et al. [29]. The deduced proteins of two of them, *MsMob1-1* and *MsMob1-4*, revealed high identity scores with characterized Mob1 sequences derived from plants, insects, animals, human, yeasts and fungi. The other two, *MsMob1-2* and *MsMob1-3*, proved to be truncated members when compared to *MsMob1-1* and were specifically expressed in flower buds during plant reproduction [29]. Genetic analysis and allelism tests of alfalfa Mob1-like members indicated that *MsMob1-1* co-segregates with the truncated *MsMob1-2/3* members and it may be the wild-type gene, whereas bioinformatic investigations suggested that *Mob1-4* is most likely a member of a different locus. Based on this, the two Mob1-like genes showing different genomic organization and position were renamed as *MsMob1-A* and *MsMob1-B*, being the former polymorphic in our alfalfa materials. Their accession numbers are AJ635582 and AM161645. Amplification of genomic DNA samples and cDNA clones with UTR-specific and ORF-specific primers confirmed the presence of two introns (393 and 105 bp) in both genes. The three exons were 283, 126 and 239 bp long and encoded for a 215 amino acid Mob1-like protein domain. Fig. 1A shows the genomic organization for *MsMob1-A* and *MsMob1-B* genes.

The *MsMob1-B* gene lacked motifs related to the promoter region at the 5′ end, but the 3′ end almost fully identified with the *MsMob1-A* gene, except for five distinct single nucleotide substitutions verified by replicated sequencing of both strands of several amplicons. The MOB1 domain of *MsMob1-B* predicted protein showed a very high similarity (99.5%) to *MsMob1-A*. The only difference was found at position 163 of the deduced amino acid sequence where a valine (codon GUU) was replaced by an isoleucine (codon AUU).

On the basis of its genomic organization, the *MsMob1-A* gene (AJ635582) most likely corresponds to the plant Mob1 protein. Database analyses revealed 88% identity and 95% similarity scores, with an E value of e-110, with the 215 amino acid AtMob1 protein (At5g45550). The *MsMob1-B* gene (AM161645) is likely related to a multi-domain protein in which the MOB1 domain is fused to one or more domains in a complex protein. Likewise, in the 1,249 amino acid AtMob1 protein (At4g19050) the MOB1 domain is combined with LRR (Leucine Rich Repeat) and NB-ARC (Nucleotide-Binding adaptor shared by APAF-1, certain R gene products and CED-4) domains. Preliminary database searches indicate the presence of a LEA (Late Embryogenesis Abundant) dehydrin-like protein domain, but the upstream region of the *MsMob1-B* gene needs further investigations.

Both *MsMob1-A* and *Mob1-B* predicted proteins scored highly significant amino acid sequence similarity in their MOB domains with Mob1 protein sequences derived from genes of various plant species. The similarity estimate ranged between 79% and 93% with predicted ORFs of an unknown function in birdsfoot trefoil, chickpea, rice, corn, Kentucky bluegrass, potato, tomato and *Arabidopsis*. It was 80% with Mob1 proteins of *Homo sapiens*, *Mus musculus* and *Rattus norvegicus*; 74% and 68% with *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* Mob1 proteins and 79%, 63% and 53% respectively with Mob1 homologues of *Drosophila melanogaster*, *Neurospora crassa* and *Caenorhabditis elegans*. The multiple sequence alignment of a subset of plant Mob1-like proteins was used to reveal conserved and variable regions among ORF sequences as well as to unveil the consensus sequence in plants (Fig. 1B).

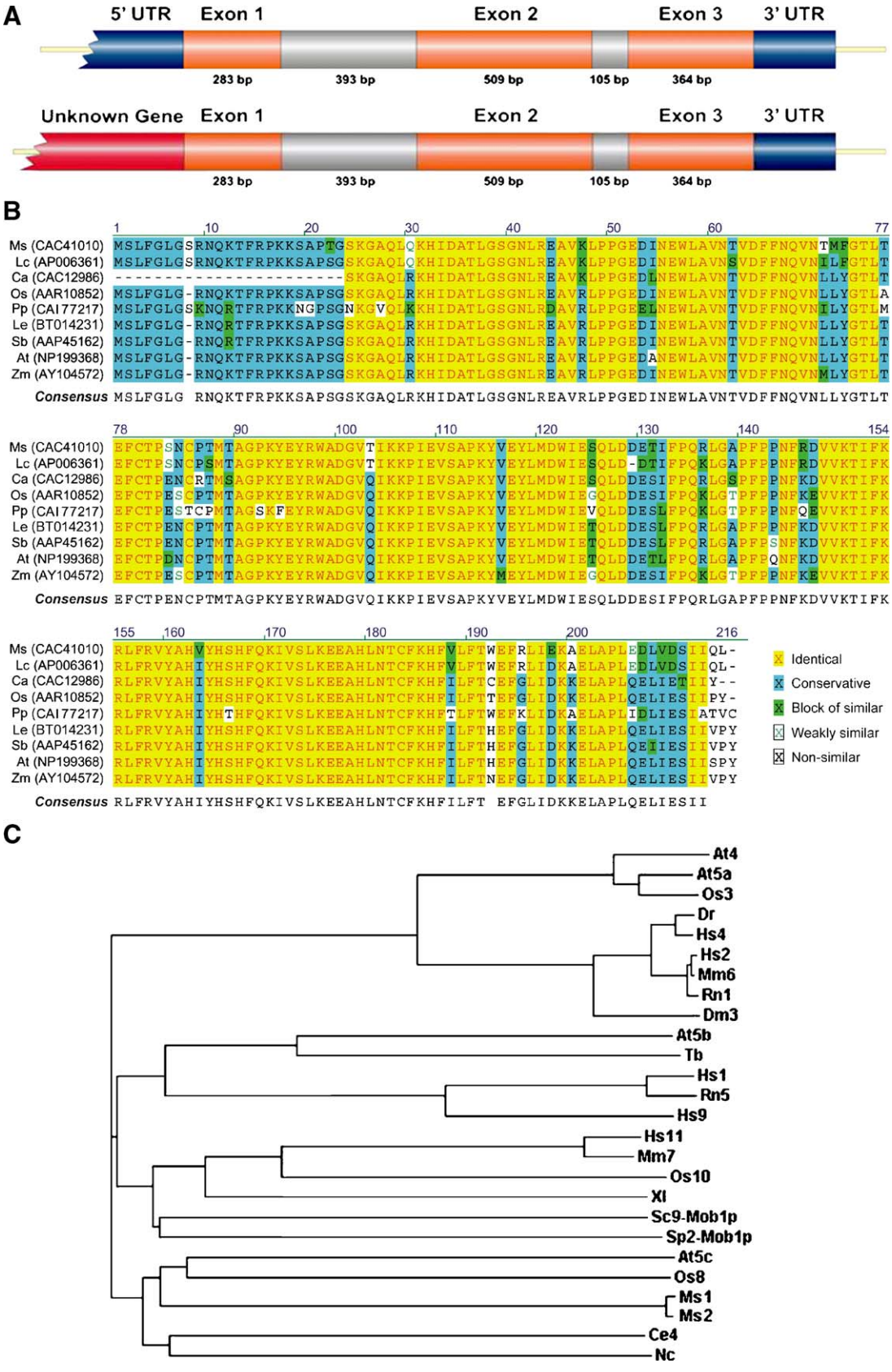
Ordination analysis performed with a number of eukaryotic Mob1 proteins selected on the basis of their pair-wise sequence similarity distinguished subgroups in which members from different organisms were tightly clustered together. The alfalfa Mob1-like proteins were clustered in a subgroup along with members of rice, *Arabidopsis*, *C. elegans* and *N. crassa*. The homology dendrogram of Mob1-like proteins from various genome databases is reported in Fig. 1C. One Mob1-like member each of rice and *Arabidopsis* were separated in distinct subgroups and similar to human and mouse Mob1 members and to a *Trypanosoma brucei* Mob1 member, respectively. Therefore, plant genomes like mammal genomes seem to contain distinct types of Mob1-like genes.

MsMob1-A and MsMob1-B genes are constitutively expressed in alfalfa organs

The expression patterns of the two Mob1-like genes were studied with semi-quantitative RT-PCR using a forward primer

Fig. 1 – (A) Organization of genomic Mob1-like genes showing position and size of exons and introns (for information on exon and intron length, sequence composition and polymorphism see results and EMBL sequence data accessions AJ635582 and AM161645); (B) multiple alignment of amino acid sequences deduced from plant Mob1-like genes recorded in public genome databases along with the consensus sequence. The Mob1-like member of *Medicago sativa* (*MsMob1-A*) shares highly significant amino acid sequence similarity with predicted ORFs of an unknown function of *Cicer arietinum* (Ca), *Lotus corniculatus* (Lc), *Solanum bulbocastanum* (Sb), *Lycopersicon esculentum* (Le), *Poa pratensis* (Pp), *Oryza sativa* (Os), *Zea mays* (Zm) and *Arabidopsis thaliana* (At). The predicted Mob1 domain of the *MsMob1-B* gene product almost fully matches that of the *MsMob1-A* gene product, except that the valine [V] in position 163 of Mob1-A is replaced by an isoleucine [I] in Mob1-B, as in other plant species. (C) Homology phylogram of Mob1-like genes constructed using the identity matrix from multiple alignments of amino acid sequences. Accessions included Mob1 proteins from distinct organisms such as plants (*Arabidopsis* chromosome 5 member, *At*NP197544; rice chromosome 8 member, *Os*AP005847), yeasts (*Sc*NPO12160 and *Sp*5978851), human cell-cycle associated Mob1A (*HsNP*775739) and Mob1B (*Hs*NP44373) proteins.

designed in a conserved region of the 3′ end in combination with a reverse primer specific for the *MsMob1-A* gene or the *MsMob1-B* gene designed in a polymorphic stretch of their 5′ end region. Primer combinations $pMob1_{For201}$/$pMob1_{Rev515}$ and $pMob1_{For67}$/$pMob1_{Rev399}$ yielded a transcript-derived fragment each of 315 bp and 333 bp, respectively. The *MsMob1-A* gene



was highly expressed in all the assayed tissues, whereas the *MsMob1-B* gene was highly expressed in roots and pods but less expressed in stems and leaves (Figs. 2A–D). The amount of its transcript was intermediate in flowers although this gene was upregulated during megasporogenesis (data not shown). RT-PCR results were confirmed by replicated experiments performed using different primer combinations. For each target gene, the amount of mRNA in all the tissues was estimated as background-corrected gross counts normalized against those of the housekeeping actin mRNA. A closer inspection of the quantitative data showed that gene expression of *MsMob1-B* was higher in roots, flowers and pods than in leaves and stems (from 2 to 3-fold times), whereas no changes in *MsMob1-A* gene expression were detectable among different tissues (see Supplementary Material).

Different isoforms of at least two distinct proteins containing Mob1-like domain are expressed in alfalfa tissues

A polyclonal antibody recognizing the peptide LGSRNQKTFR-PKKSA, located in the N terminal region of *MsMob1-A* sequence and specific of Mob1 proteins, was produced to investigate the expression and localization of these proteins in alfalfa tissues. Specificity of the antibody was tested in preliminary experiments by immunoblotting with alfalfa protein extracts. No signals were observed in replicated Western blots when the affinity-purified anti-Mob1 antibody was saturated with the correspondent peptide (1:1) (Fig. 2E).

Monodimensional Western blots revealed the presence of different Mob1-like proteins in alfalfa root tip, cotyledon and leaf extracts. A single band at 47 kDa and a doublet at about 25 kDa can be observed in Fig. 2F. According to the findings of Moreno et al. [36], showing that mammalian Mob1 cDNA, stably transfected into NIH3T3 cells, generates two distinct bands around 24 kDa, the two alfalfa 25 kDa bands can be considered isoforms of MsMob1-A protein, differing in post-translational modifications. The 47 kDa band may be instead correlated to the product of the *MsMob1-B* gene.

To further investigate the presence of different Mob1 isoforms, two-dimensional immunoblotting was performed. Fig. 2G is a representative example of 2D-Western blots obtained by using 48-h root tip protein extract. Three main spots at 47 kDa and at least three at 26 kDa and two at 24 kDa, showing different isoelectric points (pI), ranging from 6 to 8, were detected by the affinity-purified anti-Mob1 antibody. Treatment with Lambda Protein Phosphatase (λPPase), to remove phosphoryl groups from Tyr, Ser, Thr and His residues [33] revealed that most spots were phosphorylated forms (Fig. 2G, subsets 1 and 2). After 5 h of λPPase treatment, a shift of the more acidic isoforms towards a less acidic pI was detected for 24, 26 and 47 kDa proteins. This documents that phosphorylated forms of at least two proteins containing a MOB1 domain are present in alfalfa root tips.

Alfalfa Mob1 protein expression is linked to cell proliferation

RT-PCR amplification and Western blot analysis were performed on cycling and non-cycling plant organs (root tips and leaves at different developmental stages) to investigate the involvement of alfalfa Mob1 proteins in cell proliferation. The proliferating condition of the plant organs was defined with flow cytometric analysis (FCM). The distribution of the cells from each organ in the cell cycle compartments is shown in Figs. 3A–D. No DNA synthesis activity was documented in root tips after 12 h from seed imbibition and in fully differentiated leaves from the basal stem node. In both these organs all the cells were non-cycling. In fully differentiated leaves, 100% of the cells were arrested in G_0 whereas in 12-h root tips 78% and 22% of the cells were arrested in G_0 and G_2-quiescent (G_2Q), respectively. On the contrary, roots after 48 h from imbibition and newly produced leaflets from the shoot meristems contained actively proliferating cells. The percent of S-phase cells in these two organs was 31% and 12%, respectively.

Quantitative comparison of the transcripts from dividing and non-dividing root and leaf cells, detected by semi-quantitative RT-PCR with primer designed on the *MsMob1-A* gene coding region, revealed that the expression of this gene was maximal in proliferating tissues. Two-day-old root tips and young leaves displayed a markedly higher signal intensity than that of quiescent root tips and fully differentiated leaves (Fig. 3E). This result was confirmed by replicated experiments using different primer combinations (see Supplementary Material). In proliferating tissues, the *MsMob1-B* gene was expressed less in leaves than in root tips, according with the lower percent of actively cycling cells. Moreover, the quantitative difference between dividing and non-dividing cells was much more pronounced in root tips than in leaves (Fig. 3F).

As for transcripts, a marked difference in the amount of alfalfa Mob1-like proteins, assessed by 1D-Western blot image analysis, was observed between actively proliferating and quiescent tissues (Fig. 3H). Overall, the comparison of the transcripts and proteins between cycling and non-cycling root and leaf tissues was consistent with a role of alfalfa Mob1-like proteins in cell proliferation, although their presence in non-dividing cells seemed to indicate that they may have an additional function.

MsMob1 protein localization in plant meristems is cell cycle stage dependent

Mob1-like protein involvement in cell proliferation was also assayed by studying their localization during the cell cycle in synchronized root tip cells using the anti-Mob1 polyclonal antibody. The cell cycle phase was defined by quantifying the DNA content with DAPI staining and image analysis. Pre-prophase and mitotic cells were distinguished from G_2 cells on the base of chromatin and microtubule pattern. Microtubule structures were stained using a commercial anti-alpha tubulin polyclonal antibody. Qualitative and quantitative image analyses were carried out with the Image-Pro plus program (Media Cybernetics). Eight independent experiments were carried out. At least 500 cells per experiment were analyzed. As expected, most cells were synchronized in G_2-M (DNA content = 4C). The percent ranged from 40% to 60% depending on to the single experiment. About half were mitotic cells homogeneously distributed in the mitotic phases. On average, 30% of the remaining cells were distributed in G_0–G_1 (DNA content = 2C) and 20% in S-phase (2C < DNA content < 4C). Mob1-like protein distribution was stage dependent. Staining

was faint in the cytoplasm during the G_1 and S phases, but clearly visible in G_2 and during mitosis (Fig. 4A). In the G_2 phase, about 60% of the cells showed cortical microtubules. In all these cells Mob1 proteins clustered in a more or less clear ring around the nucleus. At the end of interphase, just before the onset of DNA condensation, the ring broke. The proteins

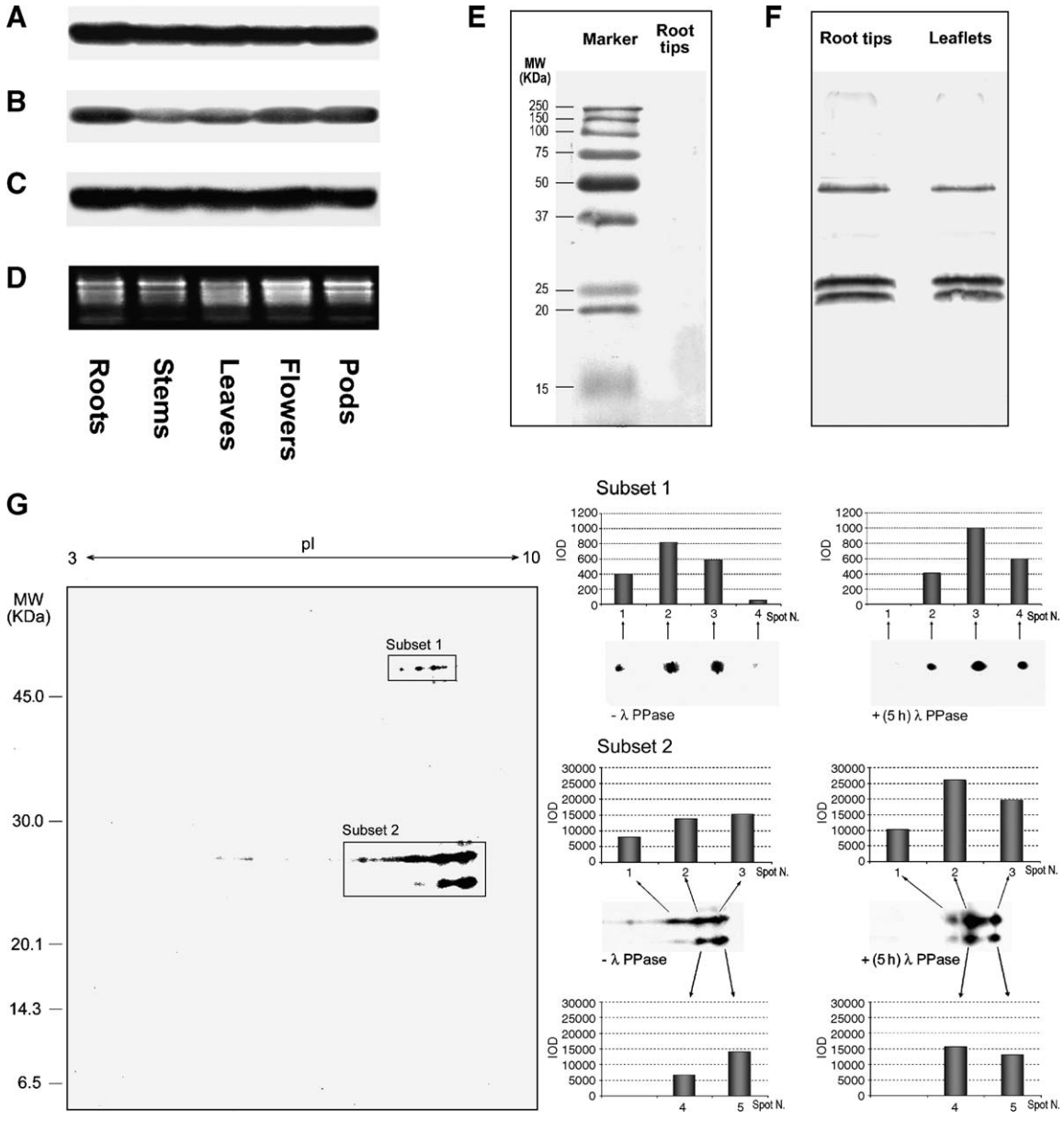


Fig. 2 – (A–D) Expression patterns of Mob1-like transcripts analyzed with semi-quantitative RT-PCR. The two primer combinations designed yield a transcript-derived fragment each of 315 bp and 333 bp, respectively. (A) The *MsMob1-A* gene is highly expressed in all the assayed tissues; (B) the *MsMob1-B* gene is highly expressed in roots and pods but expressed less in stems and leaves, while the amount of its transcript is intermediate in flowers; (C) constitutive control (actin mRNA); (D) ethidium bromide-stained total RNA. (E–G) Analysis of Mob1-like proteins in alfalfa tissues by immunoblotting with affinity-purified anti-Mob1 antibody. (E) Antibody specificity assay: negative control of Western blot obtained by saturating the anti-Mob1 antibody with the correspondent peptide (1:1). No aspecific signals were observed; (F) representative mono-dimensional Western blots showing the Mob1-like proteins detected in 48-h root tips (left) and in leaflets (right): a single band at 47 kDa and a double band at about 25 kDa were recognized in both tissues. The 25-kDa doublet likely corresponds to different post-translational modifications of the *MsMob1-A* protein, whereas the 47-kDa band is likely the product of the *MsMob1-B* gene. (G) Results of the two-dimensional Western blot analysis showing different isoforms of alfalfa Mob1-like proteins. Squares identify two sets of proteins at about 25 and 47 kDa with different isoelectric point (pI). The image analysis of the Mob1-like proteins in subset 1 and subset 2 after 5-h treatment with lambda protein phosphatase (λPPase) revealed a shift of the more acidic isoforms toward a less acidic pI, suggesting the presence of phosphorylated MsMob1 forms in 48-h root tips. IOD: integrated optical density.

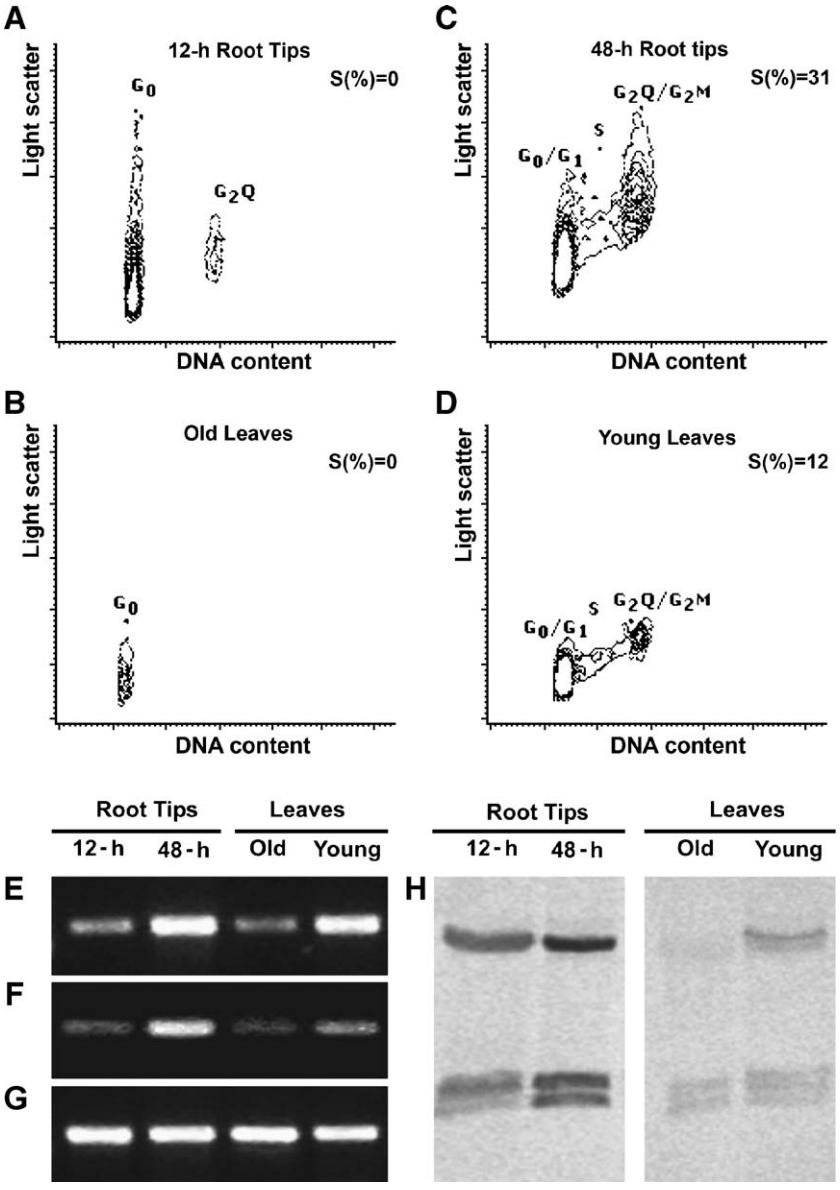


Fig. 3 – *MsMob1* gene expression in cycling and non-cycling tissues (root tips and leaves at different developmental stages) as analyzed by flow cytometry (FCM) of: (A) root tips after 12 h from seed imbibition; (B) completely developed leaves; (C) root tips after 48 h from seed imbibition; (D) newly produced leaves. (E) Results of semi-quantitative RT-PCR analysis for *MsMob1-A* gene using $pMob1_{For201}$ and $pMob1_{Rev515}$ primers. (F) Results of semi-quantitative RT-PCR analysis for *MsMob1-B* gene using $pMob1_{For67}$ and $pMob1_{Rev399}$ primers. (G) Constitutive control (actin mRNA). (H) Results of mono-dimensional Western blot analysis. G_0: 2C-quiescent cells; G_2Q: 4C-quiescent cells.

migrated to the inner border of the cell wall, thereby marking the presumed future site of cell plate coalescence. Antigens formed fairly large grains, mainly at the two opposite cytoplasmic polar regions, perpendicular to the pre-prophase band (PPB). This Mob1 pattern was evident in all the cells in late G_2 marked by PPB, although the grains located perpendicular to the PPB were not always visible likely depending on how the cell is viewed. After the start of mitosis, the antigen-related fluorescent signal disappeared and no Mob1-like protein was ever detected in the cell during prophase. The proteins might not be present or the epitope might be masked by the association with other factors or for protein conformational changes. In the next metaphase and anaphase stages, the proteins always formed cytoplasmic grains and fibrillar structures that radiated from the grains to the cell center and gently arched in the periphery (Fig. 4A). Neither structure was seen in the center of the cytoplasm, where the bipolar spindle and chromosomes were situated. From late anaphase to telophase, the grains and fibrillar structures partially converged from cell periphery to the mid-plane, between the daughter cells. The proteins were also seen in the phragmoplast region and cell plate, marking the progressive septum formation (Fig. 4A). This Mob1 protein pattern was observed in each cell traversing late anaphase–telophase.

Commercial anti-alpha tubulin polyclonal antibody was used not only to better define cell cycle phases but also to

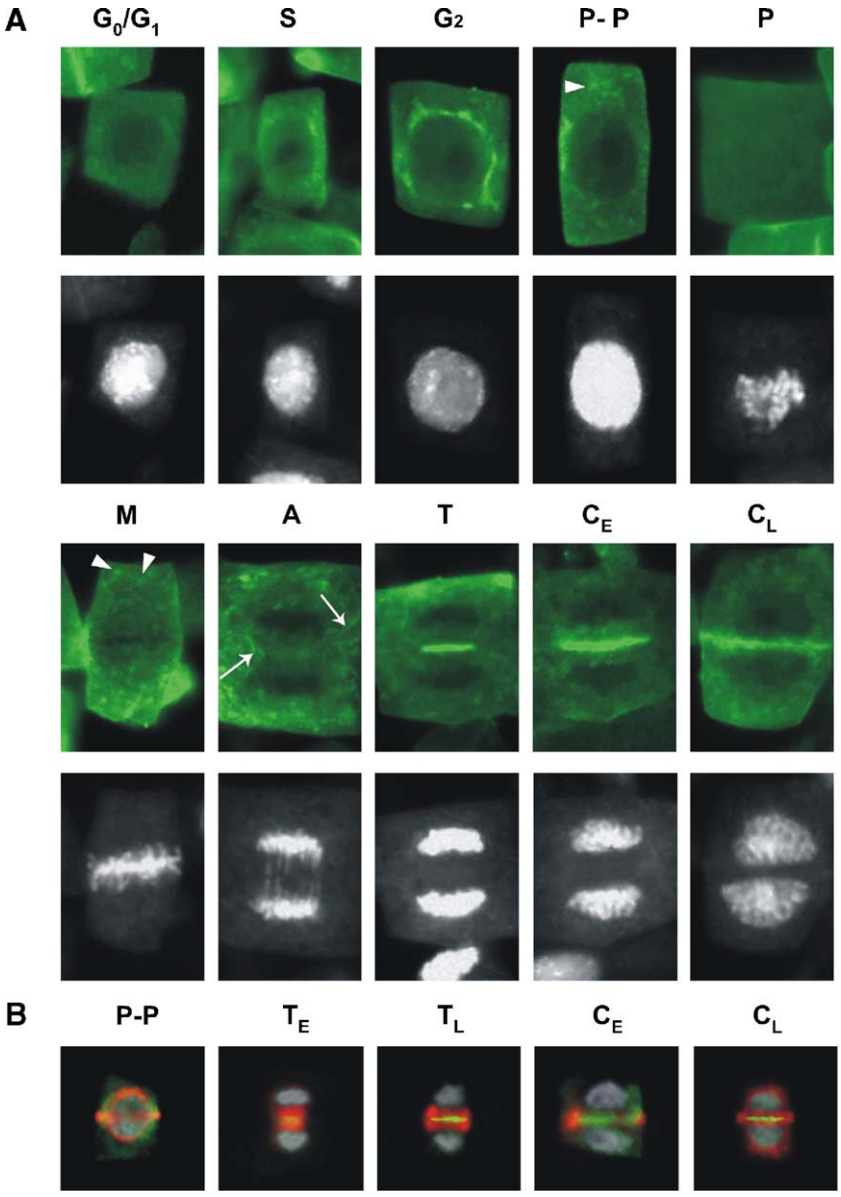


Fig. 4 – Subcellular localization of MsMob1-like proteins during the cell cycle. (A) Distribution of MsMob1 proteins in root tip cells during the cell cycle (G_0/G_1, S and G_2 stages, PP pre-prophase, P prophase, M metaphase, A anaphase, T telophase and early, C_E, and late, C_L, cytokinesis) in relation to DNA pattern, as shown by DAPI staining. The proteins form grains and fibrillar structures that are distributed in a cell cycle-dependent manner (arrowheads and arrows indicate examples of grains and fibrillar structures, respectively). In addition, they progressively mark the forming septum during cytokinesis (green signal: fluorescence related to Mob1-like proteins; white signal: DAPI fluorescence). (B) Simultaneous immunolocalization of Mob1-like proteins (green fluorescence) and alpha tubulin (red fluorescence) in cycling root tip cells during pre-prophase (PP), telophase (T) and the successive stages of cytokinesis (C_E, C_M and C_L). DNA was also stained with DAPI (white signal). The yellow signal represents tubulin and Mob1-like protein co-localization.

investigate the relationship between Mob1 proteins and microtubule structures. Image analysis showed that Mob1-like proteins partially co-localize with the PPB late in interphase (Fig. 4B). During cytoplasm partitioning, at the end of mitosis, phragmoplast microtubule and part of the Mob1-like proteins co-localized and moved from the mid-plane center to the cell periphery, so allowing and labeling septum formation (Fig. 4B). The entire cell plate still showed by Mob1 proteins staining at the end of cytokinesis, when microtubules were already newly organized as cortical structures (Fig. 4B).

Alfalfa Mob1-like transcripts and proteins are localized in the root cap meristem

Analysis of gene expression was performed in root tips by means of in situ hybridization to localize Mob1-like transcripts in alfalfa root apical meristem (RAM).

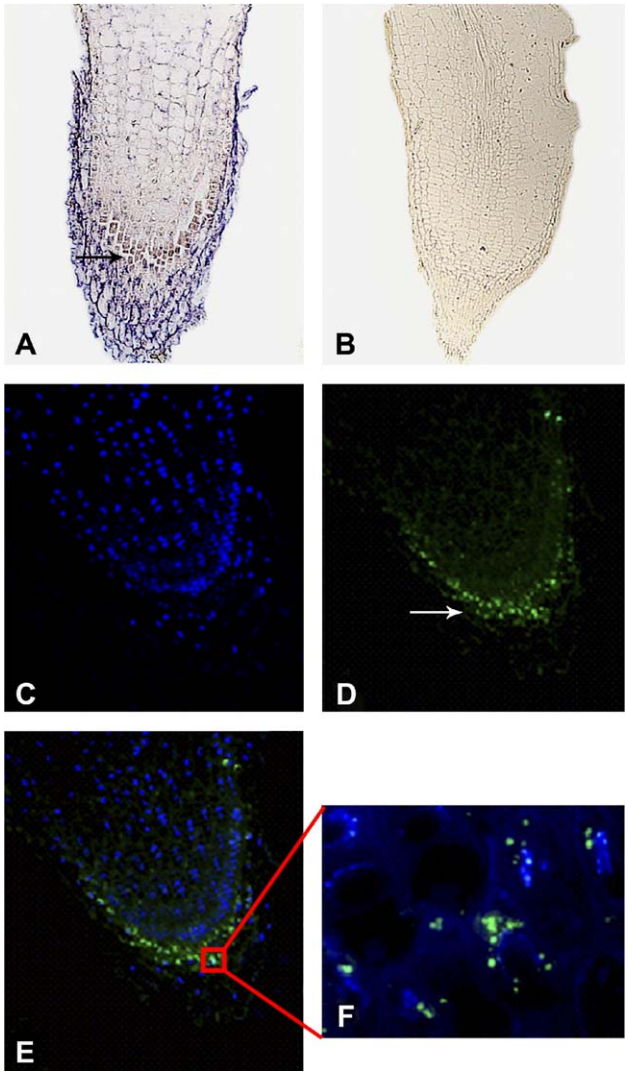


Fig. 5 – Results of in situ hybridization and immunolocalization of transcripts and proteins in alfalfa root tip sections. (A) Hybridization signals were detected in a thin cell layer of the root apex where meristematic root tip cells divide and differentiate in root cap. The blue to purple stain is the hybridization signal. (B) Negative controls obtained with the RNA sense probe. (C) Root tip section (10×) visualized by DNA staining (DAPI; blue fluorescence); (D) MsMob1 protein distribution in root tip. The Mob1 signal (green fluorescence) was specifically detected in root cap meristematic area. (E) Merge of panels C and D; (F) Magnification 100× of the selected root area (□). Mob1-like proteins were localized inside the cytoplasm as large grains around the nuclei. The fluorescence signal was never seen in the distal root cap cells.

Hybridization signals were detected in a thin cell layer of the root apex where meristematic root cells divide and differentiate in root cap (Figs. 5A, B). The hybridization signal was faint and non specific in the distal root cap cells.

An example of a semi-thin section from unsynchronized alfalfa root tip probed with the anti-Mob1 antibody is given in Figs. 5C–F. As expected, the great part of meristematic cells showed only a faint labeling because most cells were in G_1 phase and mitotic stages were not seen and because the section thickness was too thin (1 μm) allowing only a clear detection of septum labeling during cytokinesis with high magnification (100×). On the contrary, strong fluorescent signals were visible in the root cap meristematic area (Figs. 5C–E). Large grains, composed of small clustered spots, were visible inside these cells around the nuclei (Fig. 5F). A fluorescence signal was never seen in the distal root cap cells.

Expression of MsMob1-A in yeast cells is unable to complement the mob1-77 mutation

To assess the functional homology between *Medicago Mob1-A* and budding yeast *Mob1* we decided to test whether expression of *MsMob1-A* could rescue the temperature sensitive growth phenotype of the yeast *mob1-77* mutant [14]. To express *MsMob1-A* in yeast cells we cloned it under the control of the galactose-inducible *GAL1* promoter in an integrative vector. After transformation of wild-type and *mob1-77* cells, clones that expressed different *MsMob1-A* levels upon galactose induction (Fig. 6A), presumably attributable to the variable number of integrated *GAL1-MsMob1-A* fusion, were isolated. Among the analyzed clones, one expressing lower levels and one expressing higher levels of *GAL1-MsMob1-A* were selected for both the wild type and the *mob1-77* strain. A plating assay followed by incubation at 25°C (permissive temperature), 28°C (semipermissive temperature), 34°C and 37°C (restrictive temperatures) showed that expression of *MsMob1-A* was not toxic to otherwise wild-type cells (Fig. 6B). On the other hand, *mob1-77* cells carrying the *GAL1-MsMob1-A* fusion were as unviable at 28°C, 34°C and 37°C as their untransformed parental strain in the presence of galactose (Fig. 6B), suggesting that *MsMob1-A* could not complement the proliferation defects due to inactivation of *ScMob1*. Interestingly, *mob1-77* cells transformed with the *GAL1-MsMob1-A* construct were healthier at 28°C than the corresponding untransformed strain in glucose-containing medium, where the *GAL1* promoter should be turned off. The reasons for this are still unclear.

High copy number plasmids bearing the budding yeast *SIC1* gene, which encodes a cyclinB/CDK inhibitor [37,38], were shown to suppress the mitotic exit defects of *mob1* mutants up to 34°C, but not their cytokinetic defects [15]. Therefore, the question was whether *MsMob1-A* could strengthen the suppressing properties of *SIC1* at temperatures higher than 34°C and/or specifically rescue the cytokinetic defects of *mob1-77* mutant cells when co-expressed with high levels of *SIC1*. However, *MsMob1-A* turned out to be unable to ameliorate at any temperature the proliferation defects of *mob1-77* cells transformed with a 2-μm *SIC1*-bearing plasmid (Fig. 6B), or to suppress their cell separation defects (data not shown).

Discussion

We isolated and characterized novel alfalfa Mob1-like genes. The resulting protein sequences closely resembled Mob1 sequences derived from plants, insects, animals, human,

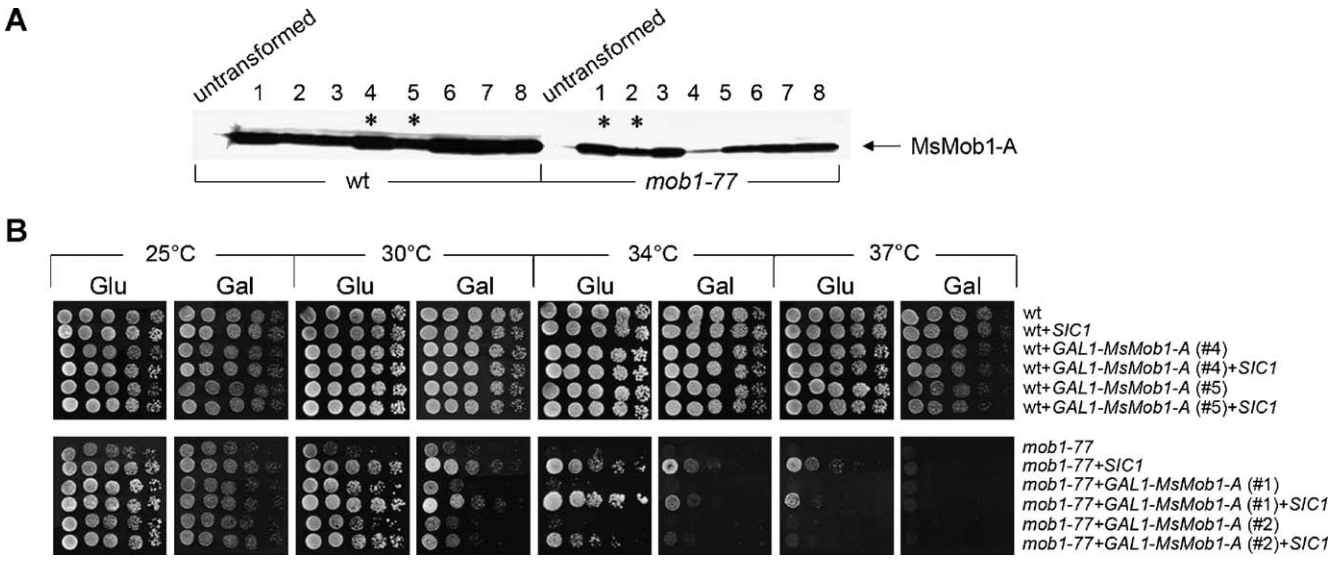


Fig. 6 – *MsMob1-A* does not complement the *Scmob1-77* mutation. (A) Western blot analysis with anti-MsMob1A antibodies of protein extracts prepared from untransformed wild-type and *mob1-77* yeast strains as well as from several transformants carrying the *GAL1-MsMob1-A* gene fusion integrated into the genome. Cells were grown overnight to exponential phase in galactose-containing medium to switch on the *GAL1* promoter. (B) Serial dilutions of isogenic strains with the indicated genotypes were prepared from cells grown to stationary phase in YEPR medium and spotted on YEP medium containing glucose (>*GAL1* promoter off) or galactose (*GAL1* promoter on). +*SIC1* indicates the presence of a *SIC1*-bearing multicopy plasmid. Plates were incubated for two days at the temperatures indicated and photographed. As shown in the upper panel expression of MsMob1-A was not toxic to wild type yeast cells, yet it was unable to rescue the growth defects of *mob1-77* mutant cells (lower panel) at restrictive temperatures. The reduced ability of high copy number *SIC1* to rescue *mob1-77* temperature sensitivity in galactose- versus glucose-containing medium may be due to differences in its expression and/or in MEN activity in media containing different carbon sources.

yeasts and fungi. A polyclonal antibody that specifically recognized the Mob1 members of the MOB gene family detected different isoforms of at least two proteins. The first one of about 25 kDa is the product of the *MsMob1-A* gene which corresponds to the plant Mob1 protein (identity and similarity scores with the AtMob1 protein (At5g45550) are 88% and 95% with an E value of e-110). The second of about 47 kDa, which is likely the product of the *MsMob1-B* gene, corresponds to a MOB1-domain containing protein.

Protein database searches revealed that the MOB1/phocein domain (pfam00931) could be combined (e.g., in *Arabidopsis* and rice) with elements of the NB-ARC domain (pfam00931), a signaling motif shared by cell death gene regulators [39], as well as with motifs of the LRR domain (pfam00560) to form complex proteins. Proteins containing a highly conserved MOB1 domain also include receptors for ubiquitination targets (F-Box), Serine/Threonine and Tyrosine kinases (catalytic domain) as well as CBL-interacting kinases. The identification of the alfalfa 47 kDa complete protein sequence is currently in progress by different molecular and biochemical approaches. Preliminary results obtained by 2D-immunoblotting analysis of immuno-precipitated proteins from alfalfa root tips followed by peptide-mass fingerprinting and LC-ESI-MS/MS have confirmed that the spots at 25 kDa correspond to *MsMob1-A* protein and shown that two distinct spots at about 47 kDa contain a MOB1 domain each associated with other unknown sequences (data not shown).

The key role of Mob1 proteins in higher eukaryotes still is poorly understood. The greater amount of alfalfa Mob1 proteins in proliferating than in non-proliferating tissues, the Mob1 proteins cell cycle-regulated subcellular localization and their presence at the cleavage site suggest that these plant proteins may have a function in cell division similar to that of yeast Mob1 essential for mitotic exit and septum formation. In yeast, the spindle pole body operates as a signaling center during cytokinesis [20]. MEN/SIN regulators such as Sid kinases and Dbf2/Mob1 temporarily associate with the spindle pole body at some point in the cell cycle. For instance, in *Saccharomyces cerevisiae*, Mob1 mobilizes to the spindle pole body (SPB) at anaphase and localizes to the bud neck, the future site for cell division, during cytokinesis [40]. In analogy to this function, centrosomes have been implicated in completing cytokinesis in animals and human cells [41]. In higher plant cells, microtubules (MTs) show dynamic structural changes during cell cycle progression and play significant roles in cell morphogenesis [42]. In addition to the cortical microtubules (CMT) that control the cell shape, the preprophase band (PPB) and the phragmoplast are other plant-specific structures which can be observed from late interphase to prophase, and from anaphase to telophase, respectively. How plant MT arrays reorganize during the cell cycle is an unanswered question. Plants lack conventional animal centrosomes and yeast SPBs and seem to possess “flexible centrosomes” from which nucleating material disperses at different cell cycle stages [43].

Despite differences between plant and yeast in mitotic entry and progression, the localization pattern in alfalfa cells of Mob1 shares many features with that of its yeast orthologue. In alfalfa cells, Mob1-like proteins form grains in the cytoplasm from which fibrillar structures radiate in all directions, preferentially toward the cell mid-plane. These grains likely correspond to sites in which microtubules are reorganized during cell cycle progression. Proteins are barely visible in G_1 and S, are clearly seen in G_2 forming a ring around the nucleus, whereas during mitosis they preferentially localize as punctuate clusters at the two opposite cellular poles. Differently from yeast, in alfalfa cells undefined Mob1 fibrillar structures are formed. In addition, during pre-prophase Mob1-like proteins mark the inner border of the cell wall in correspondence with the outer parts of PPB. In cytokinesis besides the progressive labeling of the septum, Mob1 proteins form fibrillar structures that partially co-localize with phragmoplast microtubules and partially form an aster, radiating from the growing septum poles. An interesting possibility is that Mob1-like proteins participate in cell plate orientation during cytokinesis, interacting with cytoskeletal structures and coupling the establishment of the division site, marked by PPB before the onset of mitosis, with septum formation. The interaction between MTs and Mob1p is emphasized by the characterization of haploid *mob1* yeast mutants, which display a complete increase in ploidy at permissive temperature, caused by cytokinetic defects [15]. However, although it is well demonstrated that yeast Mob1 is essential for the exit from mitosis and for septum formation, its exact function is still to be known even in this simple organism. Mob1 has been proposed to activate the mitotic exit network acting as an activating subunit of the Dbf2 protein kinase [25]. In animal cells Dbf2 homologs (NDR proteins) interacting with Mob1-like proteins have recently been discovered [26,27], suggesting a conserved function between yeast and higher eukaryotes. Dbf2 homologs have not yet been found in plants. It is unknown whether Mob1-like proteins function as kinase activating subunits during alfalfa cell division. The inability of alfalfa *Mob1-A* to complement budding yeast *mob1-77* mutation can be attributed to several reasons and therefore does not rule out the fact that the two genes do encode functional homologs. Mob1-related proteins have been proposed to act like cyclins towards CDKs, by stabilizing their associated kinases and increasing phosphorylation of their targets. It is possible that MsMob1-A does not efficiently bind to budding yeast Dbf2, which explains the lack of cross-complementation. Importantly, amino acid residues of ScMob1, such as T105, L196 and C221, that are changed in *mob1* mutant alleles and presumably crucial for Mob1 function [14,25], are replaced in a non-conservative way in the *MsMob1-A* primary sequence, suggesting that in spite of their high degree of similarity the two proteins may have substantially diverged.

Nevertheless, the involvement of higher eukaryotic Mob1 genes in cell cycle progression is supported by recent data collected in *Arabidopsis* and *Drosophila*. For instance, in *Arabidopsis* several putative cell cycle associated components, such as *AtMob1*, were targeted to the cell division plane and to the nucleus, suggesting that this organelle operates as a coordinating hub for cytokinesis [13]. Moreover, *Drosophila* Mob1 was implicated in the control of cell proliferation and apoptosis and proposed to act as tumor suppressor (Mats). In fact, its expression was capable to suppress tumor growth and lethal phenotypes induced by *mats* mutations [30]. Mob1 involvement in plant cell proliferation is also suggested by our preliminary genetic analysis using T-DNA tagged Mob1 mutants of *Arabidopsis thaliana* (locus At5g45550) obtained from the SALK Institute. Of the one-week-old F_2 plantlets assayed by PCR with specific primers, very few proved to have a double dose of the allele carrying the T-DNA. The segregation ratio observed in the progeny set was 1:1.3:0.2 being strongly deviating from the expected 1:2:1 (unpublished results). The deficit of genotypes homozygous for the insertion suggests that Mob1, which is essential for mitosis in yeast, may be required for cell cycle progression also in plants.

In plants, signaling mechanisms co-ordinate mitosis spatially and temporarily with cytokinesis to ensure integrity of genetic transfer during the cell cycle [2], and important genes required for cytokinesis have recently been discovered in plants [13]. Proteins encoded by some of these cloned genes have a cell distribution pattern similar to that of alfalfa Mob1-like proteins. This is the case of Tangled1 protein from corn [44], which is involved in orienting cytoskeletal structures during cell division, and MMK3 kinase from alfalfa [45], whose function in plant cytokinesis is still to be determined. Phragmoplastin, a dynamin-like protein from soybean, is associated with cell plate formation [46,47] but, unlike MMK3 and Mob1-like proteins, it first appears in the plate centre and as the plate grows outwards it redistributes to the growing septum margins. In *Arabidopsis*, the actin-binding protein myosin M1, the kinase Aurora and a novel cell cycle protein called T22, were targeted to the phragmoplast and shown to accumulate preferentially at the midline [13]. Finally, the syntaxin-related KNOLLE protein also locates at the cell plate and mediates vesicle fusion during cytokinesis [48]. Whereas alfalfa Mob1-like proteins appear during late telophase, when the phragmoplast reaches the lateral cell cortex, the syntaxin-related protein is present across the entire division plane.

Although based on their cellular location Mob1-like proteins may be involved in plant cytokinesis their exact function remains obscure and may include phragmoplast reorganization and orientation, vesicle transport along MTs, fusion of the vesicles at the cell plate and orientation of the septum towards the mother cell wall.

The situation is further complicated because the localization of MsMob1 proteins in the root cap may mean an additional function for this class of proteins. The root cap consists in living parenchyma cells derived continuously from the apical meristem and programmed to die. As new cells are produced in the interior, those on the root periphery are shed in an orderly manner. In plants, like in animals, programmed cell death (PCD) is a matter of life [49]. From embryogenesis to fertilization, cell and tissue death is an integral part of plant development and morphogenesis as well as response to the environment [50,51]. PCD is an active form of cellular suicide controlled by a network of genes, and the controlled initiation and execution of PCD is essential for the normal development of both plants and animals. Despite the essential nature of PCD, there are large gaps in the understanding of the

mechanistic details and molecular components controlling PCD. As reported above, our findings and database searches revealed that MOB1 domain (pfam03637) can be combined in complex proteins with elements of the NB-ARC domain (pfam00931), a signaling motif shared by animal cell death gene regulators. Proteins containing a highly conserved MOB1 domain include also receptors for ubiquitination targets (F-Box), Serine/Threonine and Tyrosine kinases as well as CBL-interacting kinases which may be implicated in either cell proliferation or cell death. As mentioned above, Lai et al. [30] recently described the growth inhibitory functions of the *Drosophila* MOB superfamily protein, termed Mats (Mob as tumor suppressor). Loss of Mats function results in increased cell proliferation, defective apoptosis and tissue overgrowth. Mob1 proteins might then turn out to be regulating both cell division and PCD also in plants . The possible involvement of MsMob1 proteins in PCD is also supported by the analysis of Mob-A and -B expression in alfalfa reproductive tissues. During megagametogenesis both transcripts and proteins were mainly visualized in reduced megaspores undergoing PCD or in the remnants of degenerated megaspores [29]. Further experiments will help clarifying the function of Mob1-like proteins in both cell proliferation and PCD. The challenge will be to dissect the roles of each Mob1-like gene in different tissues. The production and exploitation of specific antibodies against each of the Mob1-like gene products encoded by a specific member of the MOB family should aid in determining whether a multi-domain protein component with distinct functions is operative during cell proliferation and PCD.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.yexcr.2005.12.032.

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