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Is the LIM-domain Protein HaWLIM1 Associated with Cortical Microtubules in Sunflower Protoplasts ?

Authors : Christian Brière[1], Anne-Claire Bordel[1], Henri Barthou[1], Alain Jauneau[2], André Steinmetz[3], Gilbert Alibert[1] and Michel Petitprez[1]

[1]Laboratoire "Biotechnologie et Amélioration des Plantes", INP-ENSAT, BP 107 Auzeville F-31326 Castanet-tolosan, France

[2]IFR FR40, 18 chemin de Borderouge, BP 17 Auzeville, F-31326 Castanet-tolosan, France

[3]Institut de Biologie Moléculaire des Plantes du CNRS, 12 rue du Général Zimmer, 67084 Strasbourg, France

Abstract

Flowering plants express several LIM-domain proteins related to the animal CRP proteins. The expression of sunflower LIM genes was followed by RT-PCR in cultured sunflower protoplasts. A transcript was detected only for *HaWLIM1*, but not for the other two genes *HaPLIM1* and *HaPLIM2*. Polyclonal antibodies raised against either full length recombinant HaWLIM1 protein or peptides recognized a 27 kDa polypeptide on western blots. Immunocytolocalization studies showed that HaWLIM1 is located in the cytoplasm and in the nucleus. In the cytoplasm, HaWLIM1 is localized in punctate structures, which finely decorate microtubule bundles. Depolymerising microtubules with oryzalin resulted in a strong modification of the HaWLIM1 cortical pattern. In contrast, treatment of protoplasts with latrunculin B, which disrupts actin filaments, had no effect on HaWLIM1 localization. HaWLIM1 was also located within the nucleus of interphase protoplasts. During mitosis, nuclear labelling was observed in prophase, which decreased in metaphase, disappeared in anaphase, and recovered in telophase. These results suggest a dual role for HaWLIM1: in the cytoplasm, as a component of molecular complexes which may interact with microtubules, and in the nucleus, as a partner of transcription factors during interphase.

Key words: LIM-domain protein, microtubule, protoplast, sunflower, *Helianthus annuus*

Introduction

LIM proteins have been described as developmental regulators of eukaryotic, predominantly multicellular, organisms. Their name is derived from the three transcription factors LIN11 from *Caenorhabditis elegans* (Freydt *et al.*, 1990), ISL-1 from rat (Karlsson *et al.*, 1990), and MEC-3 from *C. elegans* (Way and Chalfie, 1988). They are characterized by the presence of one to five double zinc finger motifs [C-X2-C-X16-23-H-X2-C]-X2-[C-X2-C-X16-22-C-X2-3-C(D,H)] - the LIM-domains – involved in protein-protein interactions (Schmeichel and Beckerle, 1994; Dawid *et al.*, 1998).
Based on their LIM-domain structure and organization, LIM proteins fall into four major groups (Bach, 2000). The LIM homeodomain (LIM-hd) proteins, which contain two LIM domains fused to a DNA-binding homeodomain, are exclusively nuclear and act as transcription factors. The nuclear LIM-only proteins (LMO), with two LIM domains separated by a short spacer and little else, are thought to act as molecular adapter molecules and are involved in transcription (Wadman *et al.*, 1997). The LIM-kinases, which display two N-terminal LIM domains linked to a C-terminal kinase domain, participate in the regulation of cytoskeleton dynamics (Arber *et al.*, 1998; Yang *et al.*, 1998). The other LIM proteins form a structurally and functionally heterogeneous group, which comprises in particular the cystein-rich protein (CRP) subfamily.
To date several LIM-domain proteins have been described in plants including sunflower, tobacco and Arabidopsis (Baltz *et al.*, 1992; Mundel *et al.*, 2000; Eliasson *et al.*, 2000; Kawaoka *et al.*, 2000). They contain two structurally very similar LIM domains separated by a longer (40-50 residue) spacer (Eliasson *et al.*, 2000) and belong to the CRP group of LIM proteins. In tobacco, and possibly in all other higher plants, there appear to be two isoforms, one (PLIM) specific to pollen grains, and one (WLIM) expressed in all other tissues (Eliasson *et al.*, 2000). One of the sunflower proteins, HaPLIM1, was detected in the actin-enriched germination cones of mature sunflower pollen grains (Baltz *et al.*, 1999), while another protein, HaWLIM1, was found in all vegetative and reproductive organs where it was detected in many cell types and localized either to the cytoplasm, the nucleus or both. The protein was often associated with plastids in leaf cells and stem epidermis, as well as with small punctate structures in the perinuclear region of meristematic cells of root tips (Mundel *et al.*, 2000).

In this paper we report on the fine localization of sunflower LIM-domain proteins in sunflower protoplasts. The protoplast model is indeed a simple but useful system for investigating developmental patterns and early embryogenesis mechanisms in plants (Petitprez *et al.*, 1995). Our results show that sunflower protoplasts express one of the LIM-domain protein genes (*HaWLIM1*) previously identified in sunflower (Mundel *et al.*, 2000). In interphase protoplasts, the encoded protein HaWLIM1 exhibits a dual localization, inside the nucleus and in the cytoplasm where it colocalizes with cortical microtubules. In dividing protoplasts, the cortical labelling is weak and exhibits no specific organization: HaWLIM1 appears to be localized mainly in the nuclear and/or perinuclear region, at a level modulated according to the mitotic stage.

Results

Sunflower protoplasts express only HaWLIM1

Three LIM-domain protein genes have been described to date in sunflower: *HaWLIM1*,

HaPLIM1 and *HaPLIM2* (Baltz *et al.*, 1992; Mundel *et al.*, 2000; Eliasson *et al.*, 2000). We studied their expression in protoplasts by RT-PCR using primers specific for each of the three genes. An amplification product was detected for *HaWLIM1* (Fig. 1), but not for *HaPLIM1* and *HaPLIM2*, indicating that only *HaWLIM1* is expressed in sunflower protoplasts. The amplified *HaWLIM1* fragment of 565bp was directly sequenced and found to differ in only one position (nucleotide 243) from the previously reported *WLIM1* coding sequence (Mundel *et al.*, 2000); this nucleotide difference, which is most likely due to the different sunflower lines used in the respective studies (HA300 vs. EMIL), leads to a one amino acid change (N to K at position 81) in the protein.

HaWLIM1 protein polyclonal antibodies recognize a 27kDa polypeptide in sunflower protoplasts

Three different polyclonal antibodies were used in western blot experiments to detect HaWLIM1 protein in *E. coli* clones expressing *HaWLIM1* and in sunflower protoplasts: an antiserum against a GST-WLIM fusion protein, and two affinity purified antibodies (A14 and A16) against two synthetic peptides (P14 and P16) from two different regions of HaWLIM1. In bacterial extracts the anti-serum against the GST-WLIM fusion protein recognized at least 5 polypeptides, including one of about 50kDa as already described by Mundel *et al.* (2000), and one of about 27 kDa (Fig. 2A). The two affinity purified polyclonal antibodies A14 and A16 recognized only one polypeptide of 27kDa. In control blots (Fig. 2A, lane 3 and 5), the signal was either completely (A14) or partially (A16) extinct by pre-incubating antibodies with peptides P14 or P16. This indicates that A14 and A16 antibodies specifically recognize the recombinant HaWLIM1 protein.

In protoplast extracts anti-GST-WLIM antiserum recognized a 50kDa polypeptide and a 27kDa polypeptide as in bacterial extracts, and also other polypeptides (arrow head, Fig. 2B). In contrast A14 and A16 recognized only one 27kDa polypeptide and this signal was suppressed by pre-incubating the antibodies with peptides P14 or P16. The specificity of the signal was confirmed by comparison with immunoblots incubated with the preimmune serum, which recognized no proteins from protoplasts (results not shown). Thus, the three different antibodies recognize in sunflower hypocotyl protoplast a 27kDa polypeptide which corresponds to the HaWLIM1 protein.

The antiserum and purified antibodies were also used to perform immunolocalization experiments of HaWLIM1 in 1-day-old protoplasts (Fig. 3). Protoplasts incubated with antiserum (against the GST-fusion protein or against peptides) exhibited numerous fluorescent spots forming a dense network and a nuclear labelling (Fig. 3a and 3b). With affinity-purified antibodies, a similar labelling pattern was observed. However, with A14 (Fig. 3c) fluorescent spots formed a sparse dotted pattern in the cortex and chloroplasts were strongly labelled, whereas with A16 (Fig. 3d) the cortical labelling was intense, displaying clear alignments of fluorescent spots, more closely resembling the antiserum labelling pattern. The differences observed, in particular between antiserum labelling (Fig. 3b) and A14 labelling (Fig. 3c), may be due to the higher dilution of antiserum (1:2000 against 1:50 for antibody) and to a lower concentration of A14 (<8 ng/ml) than that of A16 (63 ng/ml) in the antiserum.

The specificity of the labelling was tested by incubating protoplasts with a preimmune serum or with affinity purified antibodies pre-incubated with peptides P14 or P16: in both cases they showed only a very weak diffuse fluorescence (Fig. 3e and 3f).

HaWLIM1 labelling in interphase protoplasts

Confocal microscopy observations of labelled protoplasts provided more details on the localization of HaWLIM1 during interphase. In order to compare the pattern of HaWLIM1 labelling with that of cytoskeleton networks, 3-day-old protoplasts were double-labelled with A14 or A16 antibodies and anti-β-tubulin. In the following we will present examples of labelling obtained with A16. Similar results were obtained with A14 and with the whole protein-derived antiserum.

In the cortex, HaWLIM1 spots were frequently localized along microtubules. There was a close similarity between the two patterns of HaWLIM1 and tubulin labelling (Fig. 4a-f). This can be seen clearly on Fig. 4c where most HaWLIM1 spots are localized on or close to labelled cortical microtubules. HaWLIM1 labelling decorated evenly the whole cortical microtubule network. A dense punctate labelling pattern was also observed along cytoplasmic strands and in the perinuclear region (Fig. 4d-f). Quantitative analysis of colocalization frequencies calculated on 30 protoplasts revealed that on average 78.6±1.5 % of HaWLIM1 labelling colocalized with tubulin labelling.

To provide further evidence of HaWLIM1-microtubule co-localization, 1-day-old protoplasts were incubated for 1 hour with 7 µM oryzalin in order to depolymerize microtubules. Protoplasts were then fixed and double-labelled with anti-β-tubulin antibody and the peptide directed antiserum. When MTs were depolymerized (Fig. 4h), the HaWLIM1 labelling density was weak and completely disorganized (Fig. 4g) but it could still be observed HaWLIM1 spots lying on MTs fragments (Fig. 4i). In contrast, protoplasts treated with 50 µM latrunculin B exhibited disrupted actin filaments (Fig. 4j) but neither the microtubule network (Fig. 4k), nor the HaWLIM1 pattern (Fig. 4l) were modified.

In order to estimate the frequency of nuclear labelling in relation to the mitotic stage, DNA labelling with Hoechst 33342 was performed on 3-day-old protoplasts previously immunolabelled with A16 (Total number of protoplasts observed = 178). HaWLIM1 was detected inside the nucleus of most protoplasts in interphase (92%). The labelling was either diffuse (Fig. 4m and 4n) or constituted of a dense aggregate of spots (Fig. 4o). When present, the nucleolus was not or very weakly labelled. In the perinuclear region HaWLIM1 proteins appeared to be scattered, with only a few spots lying on the perinuclear network of microtubules (Fig. 4o).

HaWLIM1 labelling in dividing protoplasts

Different patterns of HaWLIM1 labelling were observed during the progression of mitosis. The percentage of labelled nuclei decreased progressively (72% at the beginning of prophase, 25% in late prophase/metaphase, 8% in metaphase, 0% in anaphase), and increased again in telophase (100% at the end of telophase). In preprophase one could still observe a dual localisation of HaWLIM1 staining: cortical and nuclear. However, the cortical labelling appeared as scattered spots which did not seem to present any organization. Only a few fluorescent spots lay on or alongside the preprophase band (PPB) when present (Fig. 5a and b). After nuclear envelope breakdown HaWLIM1 labelling was essentially distributed around the mitotic apparatus, as seen in wide field microscopy (Fig. 5c). At metaphase, confocal sections showed mainly a high density of fluorescent spots surrounding the mitotic spindle (Fig. 5d) and only few spots inside or on the spindle. In anaphase/telophase only a weak and diffuse fluorescence could be detected in the nuclear region by wide field microscopy (Fig. 5e). Confocal observations clearly showed that this fluorescence was restricted to sparse spots around the spindle and the early phragmoplast (Fig. 5f). In telophase, a punctate fluorescent pattern was observed at the periphery of the phragmoplast. At the end of mitosis, daughter

nuclei recovered a strong labelling and the cortical labelling re-appeared (Fig. 5g and h).

Discussion

In a previous work (Mundel *et al.*, 2000), polypeptides detected in various tissues with anti-HaWLIM1 antibody had variable molecular weights, ranging from 34kDa to above 50kDa. It was then suggested that HaWLIM1 was able to form oligomeric structures. In protoplast extracts, the same antiserum detects a major band at about 52kDa and several minor bands at lower molecular weight, in particular one at 27kDa. The only polypeptide detected using antibodies directed against specific peptides (P14 and P16) has also a molecular weight of 27kDa. This weight is close to the theoretical weight of HaWLIM1 (21kDa) and this small difference in weight may be easily explained by post-translational modifications: scanning the protein sequence against PROSITE revealed three potential glycosylation sites (one in each LIM domain and one in C-terminal) and four potential phosphorylation sites (one in each LIM domain, one in N-terminal and one in C-terminal). More difficult to explain is the difference between results obtained with the antibody against the whole protein, and with peptides directed antibodies. On one hand, it is not unlikely that the antibody directed against the purified GST-fusion protein has a weaker specificity than peptide-directed antibodies. On the other hand, Mundel *et al.* (2000) have shown that this antibody recognizes specifically a 50kDa polypeptide in all tissues tested. But the essential result is that in hypocotyl protoplasts, all antibodies detect the same 27kDa protein band. Thus, without excluding the possibility of multimeric forms of the protein, our results show that HaWLIM1 is present as a monomer in hypocotyl protoplasts.

Immunolabelling clearly indicates that HaWLIM1 exhibits a dual nuclear and cytoplasmic localization in protoplasts. These two localizations appear to be common to at least two groups of LIM proteins: (i) the CRP group including the plant homologues, and (ii) at least some of the non-homeodomain, non-CRP proteins such as zyxin and LPP. Localization of LIM proteins depends on cell differentiation status. For instance, in undifferentiated fibroblasts, CRP3/MLP was detected exclusively in the nucleus, while in differentiated myotubes it is present in the nucleus and in the cytoplasm (Arber *et al.*, 1994 and 1996). In contrast, the nuclear phase of zyxin and LPP is transient and can only be detected indirectly, by using nuclear-targeted antibodies (Nix *et al.*, 1997), or by blocking nuclear export as in the case of the LPP protein (Petit *et al.*, 2000). The shuttling of these normally cytoplasmic proteins to the nucleus and back to the cytoplasm is considered to be an example of cytoplasmic-nuclear communication.

In sunflower, a dual localization of the protein had already been reported in various sunflower tissues (Mundel *et al.*, 2000). Our results on hypocotyl protoplasts indicates that the location of HaWLIM1 varies according to the developmental stage of the cell. In interphase protoplasts, labelling was seen in both the nucleus (but not in the nucleolus) and the cytoplasm. In dividing protoplasts, the labelling was essentially nuclear. In contrast to previous observations by Mundel et al. (2000), the phragmoplast was only weakly labelled by antibodies A14 and A16. This may be explained by a different specificity between affinity purified peptide-directed antibodies and the antiserum against the whole recombinant protein used by these authors. Furthermore, the latter recognizes several polypeptides, possibly heterodimers, which may have a different location from that of the monomeric form of the protein.

The localization of HaWLIM1 in punctate structures located in the perinuclear region and in the cortical cytoplasm appears to be common to plant LIM proteins. A punctate pattern for HaWLIM1 had been observed already in meristematic cells of sunflower root tips (Mundel *et al.*, 2000) and earlier studies on the pollen-specific protein HaPLIM1 had shown that the

protein accumulates in a limited number of small spots in microspores and in a dot-shaped pattern in germination cones of mature pollen grains (Baltz *et al.*, 1999). In sunflower protoplasts, a strong colocalization of the protein with microtubules was observed. This colocalization was particularly clear with cortical microtubules of interphase protoplasts. It is worth noting that the best association between HaWLIM1 and cortical microtubules was observed in 3-day old protoplasts (see Fig. 4), when they have already reorganized their cytoskeleton and rebuilt a cell wall (Petitprez *et al.*, 1995), and thus cannot be ascribed to the stress involved in protoplast generation. In animal cells, CRP proteins are known to be associated with actin-based structures. CRP/MLP proteins have been detected in focal adhesion sites and along stress fibres in cultured fibroblasts (Louis *et al.*, 1997; Sadler *et al.*, 1992) and are thought to participate in the organization of the actin cytoskeleton. In cardiac muscle MLP has been found to concentrate in the Z bands separating the sarcomers and it is believed that this protein acts in the anchoring of actin filaments to these sites. Similar sarcomer localization was described in the case of the Drosophila MLP proteins (Stronach *et al.*, 1999). Furthermore, the animal CRP and MLP proteins were shown to interact with zyxin and alpha-actinin (Louis *et al.*, 1997; Pomiès *et al.*, 1997; Sadler *et al.*, 1992), two essential components of the actin cytoskeletal architecture. To our knowledge, the only LIM domain protein known to associate with the microtubule cytoskeleton is paxillin. In human T lymphoblasts, paxillin localizes to sites of cell-matrix interaction as well as to a large perinuclear region corresponding to the microtubule organizing centre, where paxillin colocalizes with α- and γ-tubulin (Herreros *et al.*, 2000). In plants, it has been reported that a γ-tubulin-related polypeptide shows a punctate localization in cortical microtubule arrays and in the preprophase band (Liu *et al.*, 1993 and 1995), as does HaWLIM1. In sunflower protoplasts there is evidence for two γ-tubulin isoforms with a dual cortical and nuclear localization (Petitprez *et al.*, 2000). However, there is no homology between HaWLIM1 and paxillin. Furthermore, scanning the PROSITE database did not reveal the existence of any tubulin-binding domains in HaWLIM1. It is thus difficult to advocate a direct association between HaWLIM1 and tubulin.

The apparent association between HaWLIM1 and microtubules might result from the presence of fine actin filaments running alongside cortical microtubules. But disrupting the actin with latrunculin B had no effect on this pattern, which, on the contrary, was completely disorganized by microtubule depolymerization with oryzalin. Thus, such explanation cannot account for the MT-like staining pattern. From another point of view, a vesicular transport of the protein along microtubules cannot be excluded and would be consistent with the observed punctate pattern and its disorganization by microtubule disruption.

The nuclear localization of HaWLIM1 and its variation during the cell cycle suggest a role in gene transcription. It is known indeed that not only LIM homeodomain proteins (Wadman *et al.*, 1997), but also members of the CRP-type group of LIM proteins participate in gene transcription, as is exemplified by the CRP3/MLP protein (Kong *et al.*, 1997). Furthermore, in tobacco, the LIM protein NtLIM1, an HaWLIM1 homolog which is involved in lignin biosynthesis, has been shown to transiently activate the transcription of a β-gluruconidase reporter gene (Kawaoka *et al.,* 2000). However, a typical plant transcription factor generally contains a DNA-binding region and a nuclear localization signal (Liu *et al.*, 1999), two elements which appear not to be present in HaWLIM1, as revealed by sequence analysis with PROSITE. Nevertheless, several potential sites of phosphorylation were identified, which could play a role in the nuclear transport of the protein and/or in its regulation (Vandromme *et al.*, 1996; Whitmarsh and Davis, 2000). The strong decay of the protein observed at the transition metaphase/anaphase is also consistent with a phosphorylation-dependent proteolysis, and it is known that degradation of transcription factors or their coregulators is an important mechanism of transcriptional regulation (Pahl and Baeuerle, 1996). Thus, instead of

being a transcription factor *stricto sensu*, a possible role for HaWLIM1 might be to participate to the regulation of a transcription factor complex, like for instance FHL3, a LIM-only protein which interacts with CDC25B2 phosphatase in the nucleus (Mils *et al.*, 2003).
In conclusion, the data on the sunflower LIM-domain protein HaWLIM1 described in the present paper are significant at several levels: they show that the protein (a) is present in hypocotyl protoplasts, (b) exhibits a cell cycle-dependent cytoplasmic and nuclear localization, suggesting a role in transcriptional regulation. Moreover, even if more work will be necessary to fully demonstrate this hypothesis and to characterize the partner(s) of the protein, our observations suggest the existence of a, may be indirect, interaction between HaWLIM1 and the microtubule network.

Materials and Methods

Plant material

Seeds of sunflower (*Helianthus annuus* L., genotype EMIL Pioneer France Maïs) were sterilized in 5% calcium hypochloride for 20 min, then rinsed in sterile water and cultivated on MS medium (Murashige and Skoog, 1962) at 25°C under a 16-hour photoperiod (light intensity 25µE.m^{-2}.s^{-1}). Protoplasts were isolated from 8-day old plantlet hypocotyls according to the protocol of Chanabé *et al.* (1989). After purification, protoplasts were embedded at a final density of 1.5 10^5.mL^{-1} in TLD medium (Chanabé *et al*., 1991) containing 0.5% Sea Plaque agarose (FMC Bioproduct, Rockland, USA), and before solidification small drops (40 µl) of this mix were spread on poly-L-lysine (Sigma)-coated coverglasses. After solidification of the drops, embedded protoplasts were submerged in 1mL of TLD medium and cultured in the dark at 25°C.

RNA isolation

RNA isolation was performed according to Shepherd *et al.* (1983) with a few modifications. Protoplasts were ground in extraction buffer (25 mM sodium citrate, 5 M guanidium thiocyanate, 0.5% sarcosyl, 2 mM EDTA, 5% □mercaptoethanol, 1% SDS, pH 7.0). After phenol/chloroform (1:1) extraction, the aqueous phase was isolated by centrifugation (10 min at 9000 x g and 4°C) and the nucleic acids were precipitated overnight at -20°C by adding 0.05 vol. 1M acetic acid and 0.7 vol. absolute ethanol**.** After centrifugation (10 min at 9000 x g and 4°C), RNAs were specifically precipitated by addition of 2M lithium chloride, then mixed with 3 M sodium acetate and incubated for 2 hours at -20°C in 70% ethanol. Next, RNAs were dried and resuspended in sterile water. Residual DNA was eliminated by treatment with DNAse (DNAse RNAse free RQ1, Promega) according to the recommendations of the provider. Enzymes were removed by phenol/chloroform (1:1) extraction.

RT-PCR expression studies

First strand cDNAs were synthesized from protoplast mRNAs using the "Advantage RT for PCR" kit (Clontech). The following specific oligonucleotide primer sets were used to detect possible transcripts for the three sunflower proteins: 5'-ATGGCATTTGCAGGCACT-ACCCAGAAATGCATGGC-3' and 5'-TCAAGGTGCGGTTTCTCCGACCCTTGCAGAAC-GCT-3' for HaWLIM1, 5'-ATGAAATCATTCACAGGAACAACC-3' and 5'-GAAACTTCA-TCATATCGAGCTATTA-3' for HaPLIM1, and 5'-TGATTGTTGGTTCTGCCCAACTA-3' and 5'-CAATGACATACAACAGTTGTATAGACA-3' for HaPLIM2. The initial denaturation step (95°C for 5 min) was followed by 35 amplification cycles (95°C for 1 min;

50°C for 1 min; 72°C for 2 min) and a final elongation step (72°C for 15 min) in a Perkin Elmer thermocycler (Applied Biosystems). PCR products were analysed by agarose gel (0.8%) electrophoresis and then sequenced by Genome Express (Grenoble, France).

Antibodies

Three different polyclonal antibodies were used for immunodetection and immunocytology. HaWLIM1-antiserum against the GST-fusion protein was obtained as described in Mundel *et al.* (2000). An antiserum and two other antibodies (A14 and A16) were obtained by CovaLab (Lyon, France). They were directed against two synthetic peptides designed in regions of the protein non homologous to other LIM proteins: one (P14) in the C-terminal part (CLEGERSARVGETA), the other one (P16) in the inter-LIM domain (CDQLFKKTGSLDKSFE). After synthesis and purification (up to 85%) these two peptides were bound to KLH carrier protein and mixed with Freund's adjuvant. One rabbit was immunized with this mix by three intra-dermal injections at three-week intervals and one subcutaneous injection at day 63. The immune serum was obtained after 74 days. The titre of the antiserum was assessed by ELISA test and amounted to 32000. A14 and A16 antibodies were obtained by affinity purification of the immune serum against peptides P14 and P16.

Expression of HaWLIM1 in recombinant bacteria

An *E. coli* clone expressing the GST-HaWLIM1 fusion protein was obtained as described in Mundel *et al.* (2000). Protein production and purification of the GST-fusion protein on GSH-Sepharose beads was carried out with the GST Gene Fusion System (Pharmacia Biotech). After purification, cleavage of HaWLIM1 from GST was achieved using thrombin.

Western blot analysis of HaWLIM1 expression

Protoplasts were suspended in Laemmli (1970) buffer containing 4%(v/v) β-mercaptoethanol and 2%(w/v) polyvinyl-polypyrolidone. They were lysed by sonication (Vibra Cell, Bioblock Scientific) and centrifuged for 15 min at 15000 x g to eliminate cell fragments. Total proteins in the supernatant were precipitated at -20°C by adding 3 vol. 80% acetone, and the insoluble fraction washed three times in 80% acetone. Proteins were separated by SDS-PAGE in denaturating conditions by migration in a 12 % acrylamide gel. After migration, proteins were transferred onto a nitrocellulose membrane (Hybond-C Extra, Amersham). Membranes were incubated for 2 h with anti-HaWLIM1, A14 or A16 antibodies in PBS buffer containing 0.1% Tween 80 and 1% milk powder. After three washes in the same buffer, membranes were exposed to anti-rabbit IgG goat antibodies coupled to RHP peroxydase (Promega) diluted to 1:5000 and revealed by chemiluminescence (ECL kit, Amersham). Controls of specificity have been done by pre-incubating A14 and A16 antibodies with corresponding peptides (1:200) for 2h.

Immunolocalization

Protoplasts were fixed for 1 h in MSB buffer (Traas, 1989) supplemented with 2% paraformaldehyde and 0.03% glutaraldehyde. Proteins were stained according Caumont *et al.* (1997) by overnight incubation at room temperature with either rabbit HaWLIM1-antiserum (dilution 1:5000), or antiserum against peptides P14 and P16 (dilution 1:2000), or affinity purified primary antibodies A14 (dilution 1:100) or A16 (dilution 1:50). In the case of double labelling, mouse monoclonal antibody (Amersham) directed against β-tubulin (dilution 1:125) was added. After washing, protoplasts were incubated for 2 h at room temperature with FITC-labelled anti-rabbit IgG antibodies (dilution 1:50) and with Texas Red labelled anti-mouse IgG antibodies (dilution 1:50) from Amersham. After rinsing, coverglasses were mounted in an anti-fading buffer (Citifluor, Link Analytical). For negative controls, the same procedure

was followed but without the addition of primary antibodies or by adding the preimmune serum in place of the primary antibodies at the same concentration. Specificity controls were made by pre-incubating the primary antibodies A14 (dilution 1:100) and A16 (dilution 1:50) with corresponding peptides P14 (2.3 mg/mL) and P16 (6 mg/mL) respectively for 2h before immunolabelling.
In some experiments, protoplasts were pre-treated with oryzalin or latrunculin B, which are known to depolymerize microtubules (Galway and Hardham, 1989) or to disrupt actin filaments (Gibbon et al., 1999) in plant cells, respectively. One-day-old protoplasts were incubated with 7µM oryzalin or 50µM latrunculin B for 1h. They were then fixed and double labelled as described above. Disruption of the actin network was checked by indirect immunolabelling with mouse monoclonal antibody against actin (dilution 1:125) and Texas Red-conjugated anti-mouse IgG antibody (dilution 1:50) from Amersham.
Nucleus staining was performed by incubating protoplasts in an aqueous solution of 0.2µg/ml Hoechst 33342 (Sigma) for 10 min after the immunolabelling treatment.

Microscopy and image analysis

For conventional fluorescence microscopy, observations were made with a Leitz Laborlux microscope (Leica, Heidelberg, Germany) equipped with epifluorescence illumination (for Hoechst: excitation filter BP340-380, emission filter LP440, for FITC: excitation filter BP450-490, emission filter LP520, and for Texas Red: excitation filter 530-560 nm, emission filter LP580). Images were acquired using a PL APO 40x/1.32 oil objective lens (Leica) .
Confocal observations were made with a TCS SP2 confocal laser microscope (Leica, Heidelberg, Germany). Image acquisition was achieved with a HCX PL APO 40x/1.25 Leica objective lens in scan mode and image averaging of 3 frames. For FITC the 488 nm line of an argon laser was used and the emission window set to 510–530nm. For Texas Red, the 543nm line of a Helium/Neon laser was used and the emission window set to 600-700nm.
Quantification of colocalization of HaWLIM1 with tubulin was carried out on confocal sections of double-labelled protoplasts. For each section two confocal images (one for HaWLIM1 labelling, the other one for actin or tubulin labelling) were recorded in sequential mode to avoid cross talking. Images were analysed with image processing software ImagePro Plus (Media Cybernetics, Silver Spring, USA). After thresholding the images to eliminate background fluorescence, a region of interest (ROI) was carefully hand-designed inside the protoplast area, excluding chloroplasts and the nuclear region. Then, the colocalization coefficients were calculated according to the method described by Manders *et al.* (1992). Results presented are mean percentages calculated on 30 image pairs analysed in the same conditions.

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Figure legends

Fig. 1. *HaWLIM1* gene expression in sunflower protoplasts. (P) PCR with primers specific for *HaWLIM1*, (T+) positive control with *HaWLIM1* cDNA (expected size 565bp), (T-) negative control, (M) Size markers, (T-PCR) PCR internal control.

Fig. 2. Immunodetection of HaWLIM1 proteins in total protein extracts from (A) recombinant bacteria expressing HaWLIM1 and (B) sunflower protoplasts. Lane 1: antiserum against HaWLIM1, lane 2: A14 affinity purified antibody raised against peptide P14, lane3: A14 antibody pre-incubated with peptide P14, lane 4: A16 affinity purified antibody raised against peptide P16, lane 5: A16 antibody pre-incubated with peptide P16. The predicted molecular weight of HaWLIM1 is 21kDa.

Fig. 3. Immunodetection of HaWLIM1 in 1-day-old protoplasts by wide-field fluorescence microscopy. (a-d) Protoplasts were immunolabelled with: (a) antiserum against GST-HaWLIM1, (b) antiserum against both peptides P14 and P16, (c) purified antibody A14 and (d) purified antibody A16. (e-f) Specificity of antibodies labelling: no staining was observed when the antibodies A14 (e) or A16 (f) were pre-incubated with peptides P14 or P16 before protoplast immunolabelling. Bar = 10 µm, n = nucleus, c = chloroplast.

Fig. 4. Localization of HaWLIM1 in protoplasts in interphase using confocal microscopy (excepted m and n which were acquired in wide-field microscopy). (a-f) Colocalization of HaWLIM1 with microtubules in 3-day old protoplasts double-labelled by indirect immunofluorescence with A16 (green label) and anti-β-tubulin (red label) antibodies : (a, d) HaWLIM1, (b, e) β-tubulin, and (c, f) superimposition of HaWLIM1 and β-tubulin labelling. (g-i) 1-day-old protoplast treated with 7 µM oryzalin for 1h: (g) HaWLIM1, (h) β-tubulin, (i) superimposition of (g) and (h). (j-l) 1-day-old protoplasts treated with 50 µM latrunculin B for 1h: (j) control showing actin depolymerization, (k) β-tubulin and (l) HaWLIM1 labelling of a treated protoplast. (m and n) Wide-field images of protoplasts labelled with A16 (green) and anti-β-tubulin (red) antibodies. Insert: Hoechst staining of the nucleus. (o) Confocal section through the nucleus of a protoplast double-labelled with A16 (green) and anti-β-tubulin (red) antibodies. n = nucleus, nu = nucleolus. Bar = 10 µm.

Fig. 5. Localization of HaWLIM1 in 3-day-old protoplasts at different stage of the cell cycle. Protoplasts were double-labelled by indirect immunofluorescence with A16 (green label) and anti-β-tubulin (red label), and stained with Hoeschst (inserts in c, e and g). (a, b, d, f, and h) confocal sections, (c, e and g) wide field microscopy. (a,b) Confocal sections at two different levels of a protoplast showing a preprophase band. (c,d) Protoplast in metaphase (e,f) protoplast in anaphase-telophase showing an early phragmoplast (g,h) Protoplast in late telophase showing the phragmoplast and the two daughter nuclei. Note the absence of HaWLIM1 spots at the location of the future cell plate. (h) Superimposition of 20 confocal sections of a protoplast in telophase showing HaWLIM1 labelling within the two daughter nuclei and in the cortex. Bar = 10 µm.

Figure 1

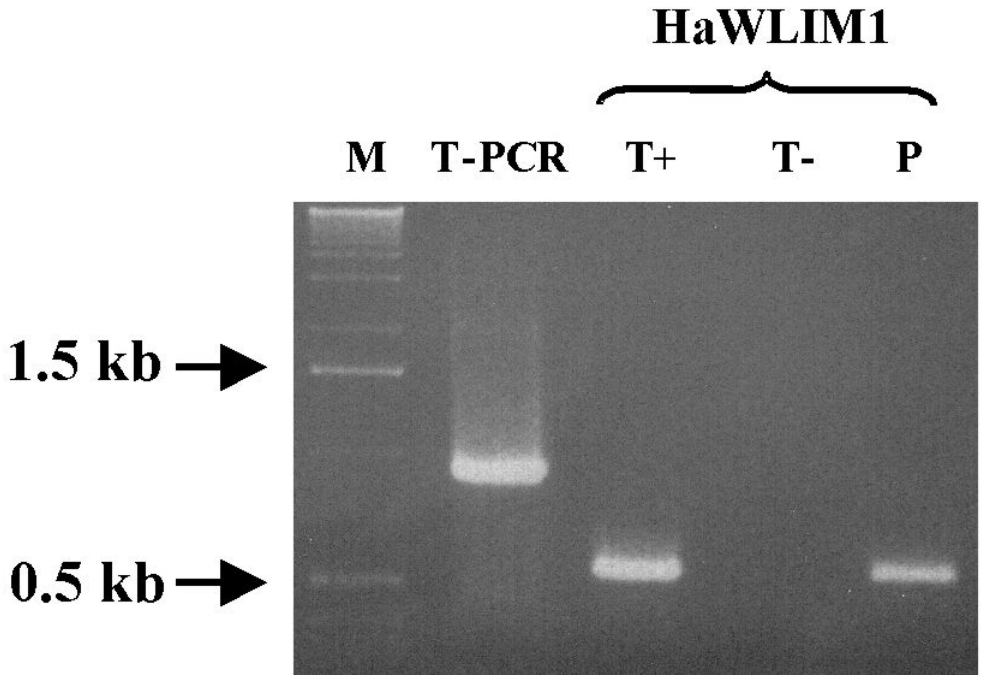


Figure 2

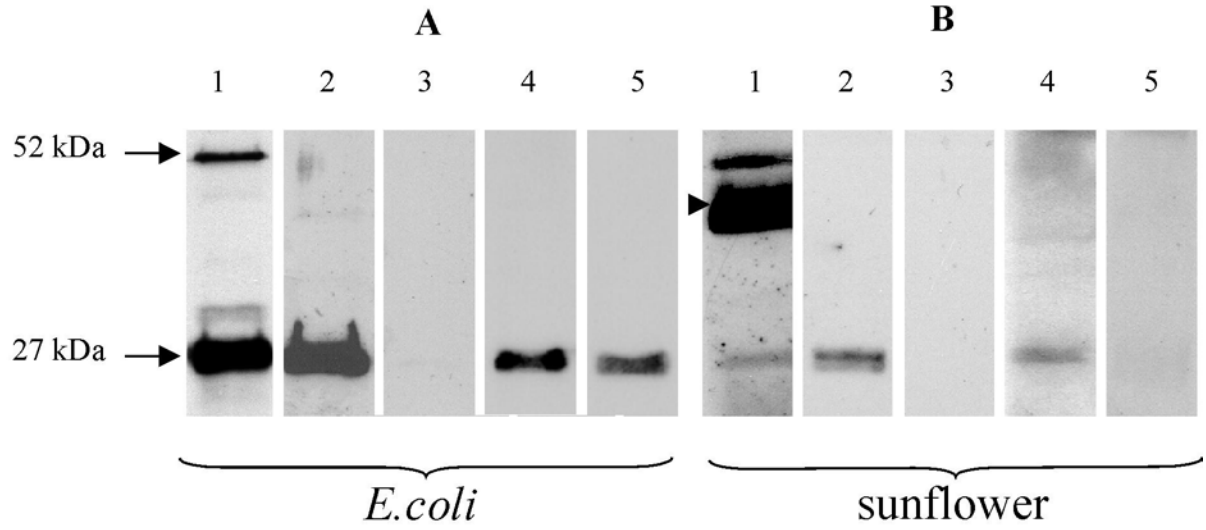


Figure 3

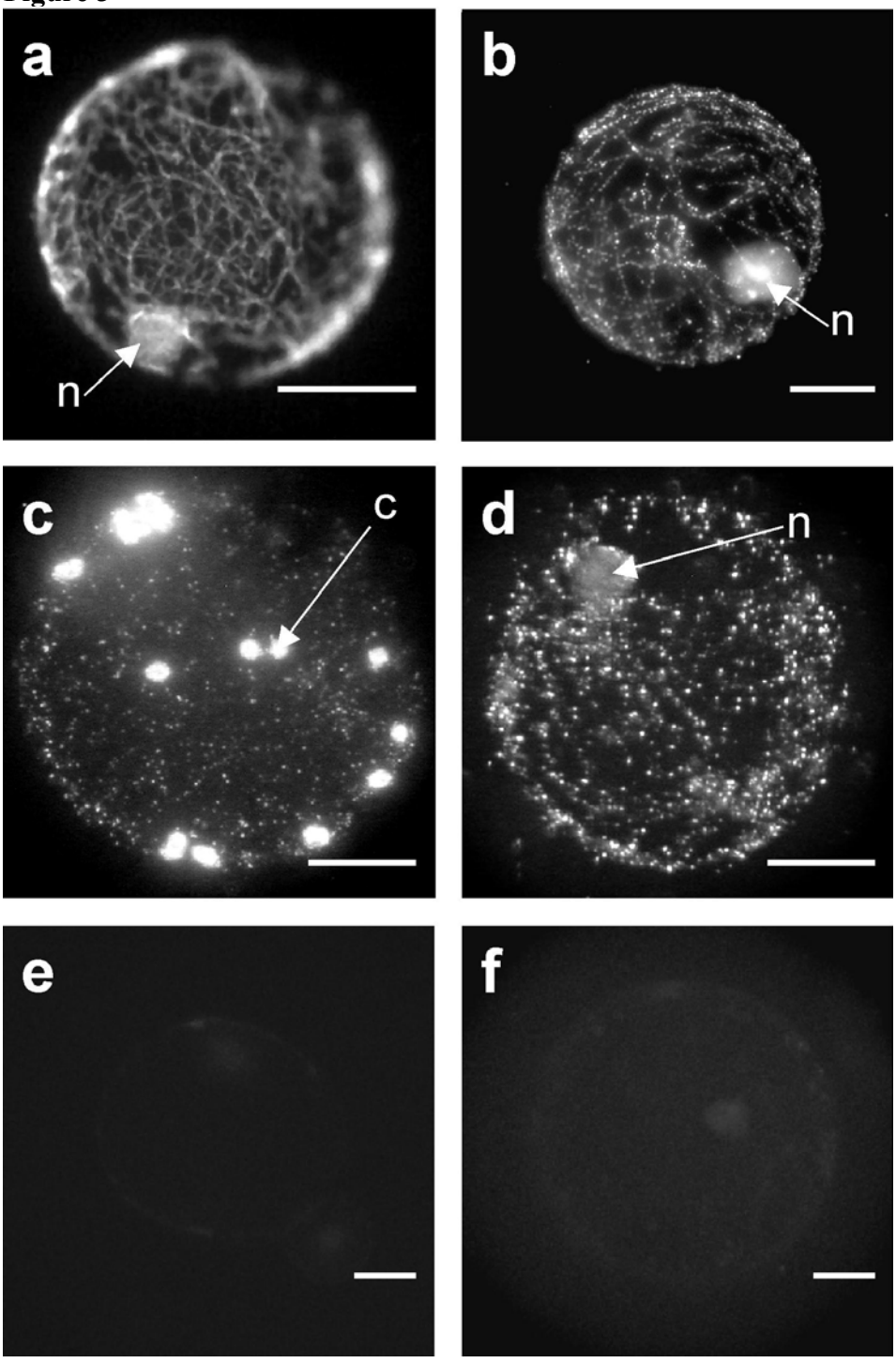


Figure 4

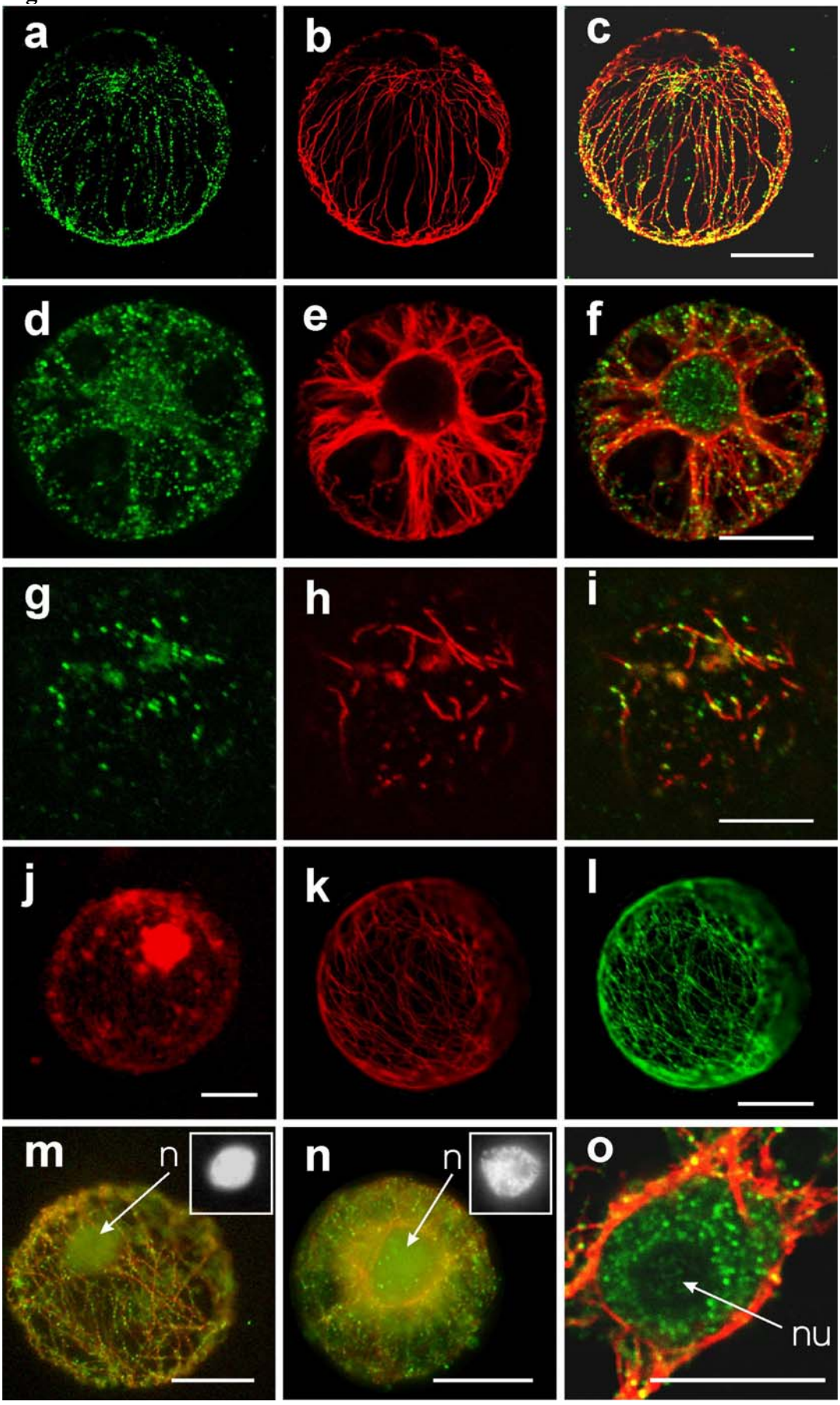


Figure 5

