DO LIM PROTEINS REGULATE THE CYTOSKELETON DYNAMICS IN SUNFLOWER PROTOLASTS ?

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Summary: The LIM protein family contains numerous proteins showing various biological functions but sharing the capacity to control the assembly and the functioning of multi-protein complexes. This property originates from the existence in their sequence of a specific structural domain, called the LIM domain. Most of the LIM proteins have been characterized in animal cells, where they are involved in the regulation of cytoskeleton elements and in the control of key developmental steps, such as cell division or embryogenesis. To date, plant LIM proteins have been characterized mainly in sunflower : in pollen (PLIM-1 and PLIM-2), and in ovaries, leaves and roots (wLIM). The biological function of these LIM proteins remains to be elucidated. In the present work, we have made the hypothesis that in plant cells, as in animal, LIM proteins could interact with cytoskeletal elements and thus could modulate their dynamics during the cell division process. Sunflower hypocotyl protoplasts were chosen as experimental model because of their ability to change their type of development according to the culture conditions.

RT-PCR experiments have shown that the wLIM gene was expressed in sunflower protoplasts, in opposition to the PLIM-1 and PLIM-2 genes which gave no amplification products. The intracellular localization of LIM proteins in protoplasts was then studied by immunocytolocalization : anti-LIM proteins antibodies labeled the intra-nuclear area and the cortical region of the protoplasts. The cortical labeling appeared as small fluorescent spots, localized along microtubules bundles. In addition, western blots have shown that anti-LIM protein antibodies recognize three different proteins in protoplasts.

Our results suggest the existence of two types of LIM proteins in sunflower protoplasts with possibly two different functions in the nucleus and in the cortical cytoplasm.

INTRODUCTION

LIM proteins are a family of regulation proteins which perform a wide range of biological functions but share a common structural domain, the LIM domain. This particular protein domain, constituted of two zinc finger motifs, is involved in protein-protein interactions. Hence, LIM proteins have the ability to interact with multiple protein partners. It is now widely accepted that these proteins mediate the assembly of multi-protein complexes and modulate the activity of some of their constituents (for reviews, see Dawid et al., 1995; Dawid et al., 1998).

The majority of LIM proteins have been characterized in animal cells where they have been localized in two different regions : the nucleus and the cytoplasm. Nuclear LIM proteins, owing to their capacity to mediate numerous interactions, play an important role in the regulation of transcription (Jurata *et al., 1996*). These proteins are involved in the control of key developmental processes, like cell division and embryonic development. Cytoplasmic LIM proteins are generally associated to the cytoskeleton. Adhesion plaques are good examples of multi-protein complexes where LIM proteins are involved (Schmeichel and Beckerle, 1994 ; Hagman *et al.*, 1998).

In plants, only a few LIM proteins have been characterized, mostly in sunflower. PLIM-1, the first LIM protein described in plants, was isolated from sunflower pollen (Baltz *et al.*, 1991 ; Baltz *et al.*, 1992). Since this observation, other LIM proteins have been characterized, such as pLIM-2 (specifically expressed in pollen) and wLIM (expressed in most of organs). The functions of these proteins remain to be determined.

Plant LIM proteins show a very high structural homology with animal LIM protein of the CRP type which are involved in protein complexes associated to the cytoskeleton (Weiskirchen *et al.*, 1995). Therefore, we hypothesized that, in plant cells, LIM proteins might interact with cytoskeletal elements and thus regulate their dynamics. Sunflower hypocotyl protoplasts were used as experimental model owing to the possibility to modulate the type of their first division, symmetrical or asymmetrical, by varying the culture conditions.

MATERIAL AND METHODS

Plant material

Protoplasts of sunflower (Helianthus annuus L., genotype EMIL) were isolated from hypocotyls of 8 day-old plantlets according to the protocol of Chanabe et al. (1989).

Extraction of total RNA from sunflower protoplasts

RNA were extracted from 100 to 200 mg of plant material frozen and stored at -80°C. Protoplasts were vortexed for a few minutes in extraction buffer (Sodium citrate, guanidine thiocyanate, sarcosyl, EDTA, β mercaptoethanol, SDS). After phenol/chloroform extraction, the aqueous phase was separated by centrifugation at 10000 rpm and nucleic acids were precipitated overnight at -20°C by adding acetic acid and absolute ethanol. After centrifugation at 10000 rpm, RNA were specifically precipitated by addition of lithium chloride with sodium acetate and incubated for 2 hours at -20°C. Next, RNA were washed in 70% ethanol, dried and resuspended in water. Residual DNA was eliminated by a treatment with DNAse (RQ1, Promega).

RT-PCR

First strands of cDNA were synthesized from protoplast mRNA with the "Advantage RT for PCR" kit from Clontech, according to the recommendations of the manufacturer. PCR reactions were carried out with DNA polymerase Gibco BRL. The initial denaturation (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 then by a final elongation phase of 15 min at 72°C (thermocycler Perkin-Elmer, Applied Biosystem). PCR products were analyzed by electrophoresis on agarose gel.

Extraction of total proteins from protoplasts

Plant material was suspended in Tris-EDTA buffer, supplemented with protease inhibitors, β mercaptoethanol and polyvinyl-polypyrolidone (Sigma). Cells were lysed by sonication and the lysates were centrifuged for 15 min at 15000 g. Proteins were retrieved in the supernatant and washed in acetone.

Western blots

Total proteins from protoplasts were separated by migration on a 12 % acrylamide gel. Proteins were then labeled by Coomassie blue or transferred on a nitrocellulose membrane. For immunodetection, membranes were incubated for 2 h with rabbit polyclonal antibodies directed against wLIM (dilution 1/30000) in PBS buffer containing 0.1% tween 80 and 1% milk powder. After two washes in the same buffer, membranes were exposed to anti-rabbit secondary antibodies coupled to RHP peroxydase (Amersham). Proteins which were recognized by antibodies were revealed by chemioluminescence (ECL kit, Amersham).

Immunocytolocalization

Protoplast were embedded into TLD solid medium containing 2% Sea Plaque agarose (FMC Bioproduct, Rockland, USA). Next, small drops (70µl) of this mixing were spread on poly-L-lysine (Sigma) coated coverglasses. Embedded protoplasts were fixed in TRAAS medium added with 2% paraformaldehyde and 0.03% glutharaldehyde and permeabilized with 0.25% triton X-100 for 30 min. Proteins were stained according to the protocol of Caumont *et al.* (1997) with primary rabbit antibodies specific of wLIM (1/15000) or primary mouse antibodies specific of β tubulin (Amersham; 1/125) associated with labeled secondary antibodies: anti-mouse IgG conjugated with Texas Red (1/50) or anti-rabbit IgG conjugated with FITC (1/50) from Amersham. For negative controls, the same procedure was followed without addition of primary antibodies. Observation were made with a Leitz Laborlux epifluorescence microscope equipped with a BP 450-490 filter for FITC and a 546/16 filter for Texas Red.

RESULTS

wLIM gene is expressed in sunflower protoplasts

RT-PCR experiments were carried out in order to study LIM gene expression in undifferentiated cells such as freshly isolated sunflower protoplasts. Specific primers of pLIM-1, pLIM-2 and wLIM were used (Fig. 1).

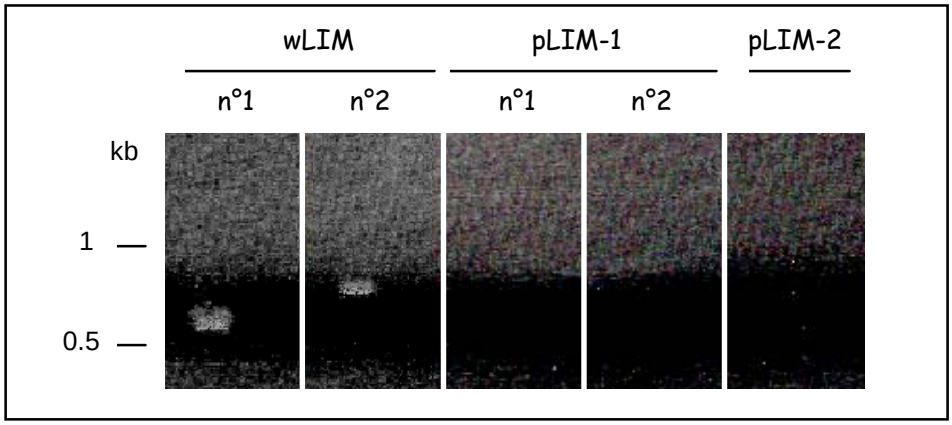

Figure 1. *Sunflower LIM genes expression in protoplasts*. PCR on the first strands of sunflower protoplast cDNA. wLIM : amplification products obtained with two pairs of primers specific for wLIM; pLIM-1 : results obtained with two pairs of primers specific for pLIM-1; pLIM-2: result obtained with one pair of primers specific for pLIM-2.

pLIM-1 and pLIM-2 genes gave no amplification products : thus, these two genes are not expressed in sunflower protoplasts. In contrast, the wLIM gene gave an amplification product whose sequence showed 96% homology with the sequence of wLIM. It is thus concluded that the wLIM gene is expressed in sunflower hypocotyl protoplasts.

wLIM protein polyclonal antibodies recognize three different proteins in sunflower protoplasts

Expression and intra-cellular localization of wLIM proteins were studied in sunflower hypocotyl protoplasts with the aid of polyclonal antibodies directed specifically against wLIM proteins. These antibodies were obtained by immunization of a rabbit against the whole wLIM protein. After migration on an acrylamide gel, total proteins were transferred on a membrane of nitrocellulose and antibodies were allowed to bind. Three different proteins of 52kDa, 62kDa and 80kDa were recognized by the antibodies.

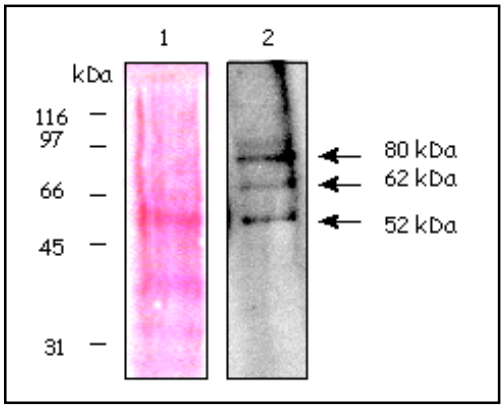

Figure 2. *Immunodetection of LIM proteins on total proteins of sunflower protoplasts*. Track 1: total proteins stained with Coomassie blue; track 2: immunodetection of LIM proteins with a polyclonal antibody directed against wLIM.

LIM proteins show a double localization in sunflower protoplasts

LIM protein localization in sunflower hypocotyl protoplasts was achieved by immunocytolocalization, with polyclonal antibodies directed specifically against wLIM associated with a secondary antibody coupled to a fluorochrome (FITC). Protoplasts were labeled at the level of two cell compartments : the nucleus and the cortical cytoplasm (Fig. 3A). The nuclear labeling was intense and diffuse (Fig. 3B), and seemed located inside the

nucleus. The cortical labeling appeared as distinct spots of fluorescence, which were more or less aligned one with each other. This type of labeling lets suppose that wLIM proteins may be located along cortical microtubules.

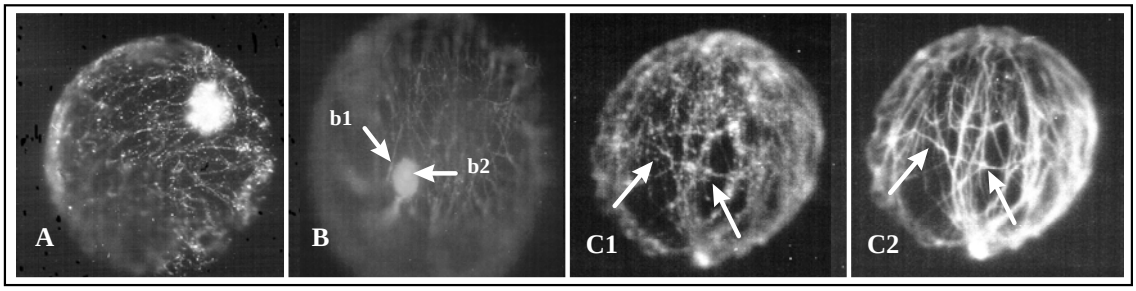

Figure 3. *Immunocytolocalization of LIM proteins in sunflower protoplasts*. Polyclonal antibodies against wLIM associated with a secondary antibody conjugated to FITC. A. LIM proteins labeling in a protoplast showing the nuclear and cortical localization. B. Nucleus labeling by wLIM antibodies: (b1) intra-nuclear labeling, (b2) nucleole; C. Double labeling of LIM proteins and microtubules on the same protoplast: (C1) protein labeling, (C2) microtubule labeling by anti-β tubulin monoclonal antibody associated to Texas Red-conjugated secondary antibody.

Double-labeling of LIM proteins and β-tubulin (Fig. 3C) gave more consistence to this hypothesis : LIM proteins appeared to constitute clusters located along cortical microtubules. The specificity of the labeling by anti-LIM protein antibodies was determined by competition experiments with unlabeled β-tubulin (results not shown).

DISCUSSION

In this work we described the expression and the localization of LIM proteins in sunflower protoplasts. Using RT-PCR techniques, we have demonstrated that, among the different LIM genes found in sunflower, only the wLIM gene was expressed in protoplasts. However, polyclonal antibodies specific for wLIM recognized three different proteins. These proteins, whose structure was found to be very close to the wLIM protein, might belong to the multigenic family of sunflower LIM proteins, which includes pLIM-1, pLIM-2, and wLIM and whose most of the members have not been yet identified (Baltz et al. 1999).
In sunflower protoplasts, these proteins showed a double intra-cellular localization: intranuclear and cortical. It is likely that these two types of plant LIM proteins fill well distinct functions in the cell. They can thus be compared to the two types of animal LIM proteins. Nuclear LIM proteins, which seem to be located inside the nucleus as shown by immunocytolocalization experiments, might be involved like their animal homologues, in the set up of protein complexes regulating transcription. In the same way, it is possible that cortical LIM proteins, located in clusters along microtubules, may be implicated in structures having similar functions as adhesion plaques in animal cells.

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