Characterization of a Meiosis-Associated Heat Shock Protein 70

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Abstract

LIM18, originally identified as a lily gene specifically expressed in meiosis I of pollen mother cells, encodes a protein which is highly homologous to heat shock protein 70. The anti-LIM18 antibody raised against recombinant LIM18 protein recognized at least three proteins (68, 69 and 70 kDa). However, two dimensional PAGE analysis revealed at least six different proteins recognized by the antibody, suggesting the existence of multiple HSP70-like proteins in lily microsporocytes. Immunofluorescence microscopy of anther tissue revealed that LIM18 immunoreactive materials are localized both in the cytosol and in the nucleus of microsporocytes. A cell fractionation experiment also suggested that LIM18 or its immunologically-related molecules is present in the nucleus. Analysis of transgenic tobacco BY–2 cells expressing LIM18–GFP fusion protein indicated the nuclear translocation of LIM18 molecule. These results suggest that LIM18 is a meiosis-associated HSP70-like protein that may be involved in nuclear and/or chromosome events during meiosis I of microsporogenesis.

Abbreviations

DAPI, 4′, 6-diamidino-2-phenylindole; GFP, green fluorescent protein; PAGE, polyacrylamide gel electrophoresis.

1. Introduction

All organisms respond to elevation of environmental temperature by synthesizing several families of proteins, known collectively as heat shock proteins (HSPs). Among these proteins, HSP70s are one of the most conservative proteins and are found in bacteria as well as humans. In eukaryotes, HSP70s are encoded by a multigene family and are classified into subgroups based on the difference of their expression pattern. Some of them are the heat shock cognate 70 (HSC70) proteins that are expressed constitutively and are involved in normal cellular processes such as protein folding, assembly and/or disassembly of macromolecular complex and intracellular targeting. Others are the inducible HSP70s which are produced under certain conditions of stress such as heat, cold and heavy-metal exposure, and are probably involved in the refolding of denatured or aggregated proteins (Mierynk et al., 1992; Hendrick and Hartl, 1993; Fernandes et al., 1994; Frydman et al., 1994; Boston et al., 1996; Hartl, 1996; Mierynk, 1997; Guy and Li, 1998; Schoffl et al., 1998). On the other hand, the expression pattern of certain HSP70 has been shown to be developmentally regulated. For example, meiosis-associated accumulation of HSP70 mRNAs or proteins have been reported in yeast, mouse and rat (Kurtz et al., 1986; Zakeri et al., 1988; Wisniewski et al., 1990; Rosario et al., 1992; Wisniewski et al., 1993). These HSP70s are thought to be involved in critical processes of the progression of meiosis.

Previously, 18 meiosis-associated cDNAs were isolated from microsporocytes of Lilium longiflorum and their corresponding genes designated as LIM (Lily messages Induced at Meiosis) genes. Among their gene products, LIM18 shows high similarity to other eukaryotic HSP70s, and its mRNA has been shown to be specifically expressed during meiosis I of microsporogenesis (Kobayashi et al., 1994).

In the present study, we characterized HSP70-like molecules expressed during meiotic progression in higher plants. Although it is generally difficult to prepare samples for the study of meiosis in higher organisms, L. longiflorum has provided an excellent
system for the study of biochemical and cytological events that are correlated with the different stages of meiosis, because these stages are protracted and synchronized in anthers (Erickson, 1948). Taking advantage of this system, we carried out a series of experiments for the characterization of a meiosis-associated HSP70-like molecule, LIM18. In this study, in order to characterize the HSP70s in meiotic cells at the protein level, we generated anti-LIM18 antibodies and performed immunological analyses. Immunofluorescence microscopic observation and cell fractionation experiments indicated the presence of LIM18-related molecules in the cytoplasm as well as in the nucleus. In addition, using the green fluorescent protein (GFP) from *Aequorea victoria* as a protein tag, intracellular targeting of LIM18 molecule was also investigated. Analysis of transgenic tobacco cells expressing chimeric proteins suggested that the LIM18 is targeted to the nucleus.

2. Materials and Methods

2.1 Plant Materials

Protein samples from floral buds of lily (*Lilium longiflorum* cv. Hinomoto) were prepared as described previously (Mousavi et al., 1999). Proteins for two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) were prepared by the phenol extraction method (Barent and Elthon, 1992).

2.2 Expression of recombinant LIM18 and antibody production

The pBluescript-LIM18 plasmid (Kobayashi et al., 1994) was digested with the *KpnI* and the fragment containing a coding region was subcloned into *KpnI* site of pQE30. Recombinant LIM18 was purified following the protocol for QIAexpress Expression System (Qiagen). Purified recombinant protein was used for immunizing rabbits (Japanese white rabbit) and mice (BALB/c mouse), and for affinity purification of the anti-LIM18 antibody.

2.3 Two-dimensional gel electrophoresis

Two-dimensional PAGE was performed by the method of O’Farrell (1975) using a Mini-PROTEAN II 2-D CELL system (Bio-Rad) according to the manufacturer's instructions. For the first dimension, the isoelectric focusing gel electrophoresis was carried out by combining Bio-Lyte 5/7 and Bio-Lyte 3/10 (Bio-Rad) in a ratio of 4:1. Second dimension SDS-PAGE was carried out using 10% acrylamide gels.

2.4 Cell fractionation

Nuclear and cytosol fractions of lily microspores were prepared based on the method described previously (Higashitani et al., 1990). Pachytene microsporocytes from 30 to 50 buds were washed three times with buffer B containing 20 mM Tris-HCl (pH 7.5), 10% glycerol, 0.2 mM ethyleneglycol bis(2-aminoethyl ether) tetraacetic acid, 0.1 mM phenylsulfonylfluoride, 0.5 mM dithiothreitol, 0.3 M sucrose, and 10 mM MgCl₂. The cell suspension was homogenized in 4 ml buffer B with a glass homogenizer, and centrifuged at 4,000g for 10 min at 4 °C. The supernatant was used as the cytosol fraction. The pellet was washed two times with buffer B and resuspended into 1 ml buffer B. Then the solution was overlaid onto 4 ml of 30% Percoll in buffer B and centrifuged at 1,500g for 10 min at 4 °C. The pellet was washed two times with buffer B and resuspended into buffer B without 0.3 M sucrose and 10 mM MgCl₂. The resultant cell suspension was used as the nuclear fraction. BY-2 cells were fractionated following the reported protocol with modification (Busk and Pagès, 1997). BY-2 cells were suspended in buffer A, (10M HEPES pH 7.8, 10mM KCl, 10mM MgCl₂, 5mM EDTA, 1mM dithiothreitol, 0.2mM phenylsulfonylfluoride, 25% glycerol), and homogenized using a glass homogenizer. The homogenate was filtered through Miracloth (Calbiochem) by centrifugation and the resultant supernatant was used as the cytosolic fraction. Pellet was washed twice with buffer A and used as the nuclear fraction.

2.5 Western blotting and immunolocalization

Antibody detection of protein samples was done as described previously (Mousavi et al., 1999). Anti-GFP monoclonal antibody (Clontech), anti-LIM18 polyclonal antibody, anti-LIM18 monoclonal antibody (7A3), anti-LIM13 polyclonal antibody, and anti-histone H2B antibody (generously gifted from Dr. Ichiro Tanaka) was used at dilution 1:2, 1:2000, 1:2, 1:500, and 1:2, respectively. The samples were immunostained as described previously (Mousavi et al., 1999).

2.6 Reporter gene constructs and transformation of BY-2 cells

For preparation of the *LIM18–GFP* construct, C-terminal coding region of LIM18 cDNA was amplified by PCR using the following primers:

5'- ACCAGATCTACCTCTCCATCCTCGGTTCC-3';  
and  
5'- TTGTTGCCAGGAAATTGGTG -3'.

The amplified fragment was digested with *BgIII* and then blunted by a fill-in reaction. The fragment
was digested with BstXI and inserted into the XbaI and XhoI-blunted site of pGFP2 together with XbaI–BstXI fragment of pBluescript–LIM18 plasmid (Kobayashi et al., 1994). The CaMV 3S promoter–LIM18–GFP region was excised by digestion with SphI and AarII and blunted. Finally, the fragment was subcloned into the EcoRI–blunted and Smal site of binary vector pBI101 (Clontech). For preparation of the LIM13::GFP construct, a XbaI/SacI fragment of the original LIM13–GFP plasmid (Ogata et al., 1999) was subcloned into the XbaI/SacI site of pBI121 (Clontech). The plasmids were transformed into Agrobacterium tumefaciens LBA4404 by the electroporation method. BY-2 cells were transformed essentially as described by Matsuoka and Nakamura (1991).

2.7 Microscopy

Intracellular localization of GFP fusion proteins and FITC-stained samples were observed using a Olympus BHT fluorescence microscope and photographed with a PM–10ADS camera (Olympus, Tokyo, Japan) as described previously (Ogata et al., 1999; Mousavi et al., 1999).

3. Results

3.1 Production of recombinant LIM18 protein

To investigate the LIM18 gene products in meiotic cells, we exploited the immunological approach using antibodies raised against recombinant LIM18 protein. A plasmid DNA for the overexpression of histidine-tagged LIM18 protein was constructed and introduced into E. coli. Induction of expression by the addition of 1 mM IPTG to the medium led to the production of a 69-kDa protein. The recombinant protein was affinity purified by Ni²⁺-NTA column chromatography. In addition to the 69-kDa protein, a number of proteins were co-purified as resolved by SDS–PAGE gel. Because none of these proteins could be purified from IPTG-induced E. coli extracts which was transformed with the vector alone, these smaller proteins seemed to be degradation products and/or its translation were stopped in the coding region prior to the stop codon of LIM18. However, it is also possible that these extra bands are bacterial proteins that bind to LIM18 protein (Fig. 1). The purified fraction was used as an antigen for anti-LIM18 polyclonal and monoclonal antibodies.

3.2 Isoform composition of HSP70s in meiotic cells

In order to investigate the expression pattern of LIM18 and immunologically related HSP70 homologs, protein samples prepared from different tissues of Lilium longiflorum were separated by SDS–PAGE. Immunoblot analysis revealed that the anti-LIM18 antibody recognizes at least three protein bands with a molecular mass of 68-, 69- and 70-kDa, respectively. The 68- and 69-kDa bands appeared to be specific in the young anther, microsporocytes and immature/mature pollen. On the other hand, the 70-kDa protein was also detected in the leaf, stem and root (Fig. 2). To identify the immunologically-related proteins, we analyzed the HSP70-like proteins expressed during microsporogenesis by 2-D PAGE analysis. As shown in Fig. 3, six protein spots reacting against the anti-LIM18 polyclonal antibody were detected in extracts prepared from the pachytene stage microsporocytes (Fig. 3). Identical results were obtained from leptotene, zygotene and tetrada cell extracts.

![Fig. 1](image1.png)  
Fig. 1 Expression of LIM18 recombinant protein in E. coli. SDS–PAGE analysis of total protein extracts from IPTG-treated E. coli cells carrying expression vector alone (lane 1), pQE30–LIM18 vector (lane 2), and affinity purified protein using Ni²⁺ resin from IPTG-treated E. coli cells carrying expression vector alone (lane 3), pQE30–LIM18 vector (lane 4). Protein molecular mass markers are indicated at the left in kilodaltons. Arrow indicates histidine-tagged LIM18.

![Fig. 2](image2.png)  
Fig. 2 Expression profile of HSP70s in L. longiflorum. Total protein (5 µg) was extracted from various tissues of L. longiflorum and separated on SDS–PAGE gel with 7.5 % acrylamide and immunostained using anti-LIM18 polyclonal antibody. Sample analyzed are follows: lane 1, young anther; lane 2, leptotene cell; lane 3, zygotene cell; lane 4, pachytene cell; lane 5, diplotene / diakinesis cell; lane 6, tetrada cell; lane 7, immature pollen; lane 8, mature pollen; lane 9, leaf; lane 10, stem; lane 11, root.
3.3 Immunolocalization of the LIM18 protein

To examine the spatial distribution and intracellular localization of LIM18 protein in lily, we conducted immunocytochemical experiments on sections of resin embedded anthers using an anti-LIM18 polyclonal antibody. As shown in Fig. 4, accumulation of LIM18-related protein is evident in metaphase I microsporocytes. Unlike the results obtained by western blot analysis, no strong signal was observed in sporophytic cells and tapetum (Fig. 4, A and B). The difference between the immunolocalization study and western blot analysis is...
3.4 Intracellular targeting of the LIM18-GFP fusion protein

To investigate the intracellular localization of LIM18 protein in the living cells, we fused cDNA encoding the full length LIM18 in-frame to the Aequorea victoria green fluorescent protein (GFP) gene. In order to obtain transgenic plant cells expressing the GFP fusion proteins, we used tobacco BY-2 cells because of the higher efficiency in Agrobacterium-mediated transformation. A meiosis-associated nuclear protein, LIM13, was used as a nuclear-localized marker protein (Ogata et al., 1999). We generated transgenic tobacco BY-2 cell lines expressing GFP, LIM13-GFP and LIM18-GFP, respectively, under the control of cauliflower mosaic virus 35S promoter. In order to confirm the transgene expression, we carried out immunoblot analysis of protein extracts prepared from BY-2 transgenic cells. Antibodies against GFP, LIM18 and LIM13 proteins were used to detect the expression of transgene products. As shown in Fig. 6, the anti-LIM13 antibody specifically recognized a LIM13-GFP protein. With anti-LIM18 antibody, in addition to a 100-kDa band of LIM18-GFP fusion protein, lower molecular weight bands were also detected even from the wild type BY-2 cells. Using the anti-GFP antibody, we could detect GFP and LIM18-GFP expression specifically.

Observation of transformed BY-2 cells by fluorescence microscope revealed the nuclear localization of LIM13-GFP as compared with GFP alone (Fig. 7). While the LIM18-GFP protein appeared to be targeted to the nucleus, unlike LIM13-GFP, the fluorescence signal was also detected in the cytoplasm.

To confirm the localization of LIM18-GFP, we carried out cell fractionation experiment using

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Fig. 6 Detection of GFP fusion proteins in transgenic BY-2 cells. Protein extracts from wild type and transformed BY-2 cells expressing GFP alone, LIM18-GFP and LIM13-GFP were analyzed by immunoblotting using anti-GFP monoclonal, anti-LIM18 polyclonal and anti-LIM13 polyclonal antibodies. Arrowhead, arrows and asterisk indicate GFP, LIM18-GFP and LIM13-GFP, respectively. Protein molecular mass markers are indicated at the left in kilodaltons.
transgenic BY-2 cells. The result obtained by protein gel blot analysis was consistent with the microscopic observation. LIM13-GFP was detected from the nuclear fraction, while GFP alone was detected mainly from the cytosol. On the other hand, LIM18-GFP was detected both in the cytoplasmic and nuclear fraction (Fig. 8).

These results strongly suggested that LIM18 is able to direct GFP into the nucleus. However, unlike conventional NLS-containing proteins, a certain amount of LIM18-GFP fusion protein was also detected in the cytoplasm. Taken together, we concluded that the LIM18 protein is dual-targeted to the nucleus and the cytosol.

4. Discussion

In higher plants, HSPs have been suggested to be involved in certain developmental processes. In addition to HSP70s, the expression of HSP90s and low molecular weight HSPs has been shown to be induced during embryogenesis, flowering and another development (Bouchard, 1990; Dietrich et al., 1991; Atkinson et al., 1993; Marrs et al., 1993; Tsukaya et al., 1993; Duck and Folk, 1994; zur Nieden et al., 1995; DeRocher and Vierling, 1995; Gagliardi et al., 1995; Futamura et al., 1999). These studies provided evidence for the gene expression of HSPs only at transcriptional levels. In the present study, we examined the protein levels of HSP70s to understand the function of meiosis-associated HSP70-like proteins, which was not obtained in earlier studies.

In order to obtain a specific antibody against LIM18 molecule, we used E. coli produced recombinant LIM18 protein as an antigen for the immunization of rabbit. However, we could not obtain a specific polyclonal antibody that could discriminate the LIM18 molecule from other HSP70-related proteins. We also tried to obtain monoclonal antibodies for the specific recognition of the LIM18 protein. As shown in Fig. 3, even with the monoclonal antibody, we were unable to distinguish the LIM18 protein from other molecules expressed in somatic tissues. These results suggest the existence of closely related multiple HSP70-like molecules in lily. Using 2D-PAGE analysis, we could detect up to six different spots reacting against the LIM18 antibody in meiocytes and three of them appeared to be microsporocyte specific. However, since phosphorylation of HSP70s has been observed in tomato cell cultures (Novo and Scharf, 1984), it is possible that these multiple protein spots detected in this study are the result of differences in the modification state of the protein. On the other hand, because at least three HSP70s were detected in mouse meiotic cells (Rosario et al., 1992), it is also possible that redundant and/or distinct functions of immunologically-related HSP70s are involved in the maintenance of microsporocytes in lily.

The cells at meiosis and later stage haploid cells have a thermostensitivity much higher than that of somatic cells. In L. longiflorum, by heat-treatment onto microsporocytes, we could not observe any significant increase of HSP70s recognized by anti-LIM18 antibody (data not shown). Furthermore, no
consensus heat shock element was found in the 1.7-kb promoter region of LIM18 (Minami et al., unpublished data). Therefore, it seems likely that
the expression of LIM18 and related proteins are not regulated by the environmental stresses but by developmental factors to act in progression of
meiosis and differentiation into gamete under non-stressed conditions. Developmental and organ-specific regulation of HSP70 gene has also been
described in other plants. For example, the expression of Hsp70s are induced during pollen development in maize and tomato (Duck and Folk, 1994;
Gagliardi et al., 1995), and cytoplasmic HSP70s are differentially expressed in vegetative and embryonic organs in pea (DeRocher and Vierling, 1995).
Although more detailed investigations are necessary, comparative studies of these HSP70s with LIM18 will be interesting.

Indirect immunofluorescent microscopy and cell fractionation experiments of lily microsporocytes demonstrated the existence of proteins that are
recognized by anti-LIM18 polyclonal antibody in the nuclei as well as in the cytosol (Fig. 4, 5). Interestingly, co-localization with chromosomes was also observed (Fig. 4). This intracellular localization feature in microsporocytes is quite similar to
that of the HSP70-2 of mouse in spermatocytes (Allen et al., 1996). HSP70-2 is a meiosis-specific member of HSP70 family and its structural feature
also shows high similarity to LIM18 (74% identity at the amino acid level). HSP70-2 has been shown to be localized along the synaptonemal complex (S. C.) in mouse pachytene spermatocytes (Allen et al., 1996), and the disassembly of the S. C. during diplotene/diakinesis phase is disrupted in Hsp70-2−/− mice (Dix et al., 1996, 1997). In addition, Zhu et al. (1997) reported that HSP70-2 interacts with cdc2 and its transient interaction is necessary for activation of cdc2 in mouse testis. Consequently, in Hsp70-2−/− mice the association between HSP70-2 and cdc2 does not occur, the progression of meiosis is stopped prior to diplotene/diakinesis, and the formation of normal sperm cells was not observed (Dix et al., 1996, 1997; Eddy, 1999). Because of its high similarity to HSP70-2 at the amino acid sequence level and its expression pattern, it is highly possible that the LIM18 protein is also involved in critical processes of meiotic progression and chromosome behavior.

We examined the intracellular localization of LIM18 by GFP tag strategy. Using a microprojectile bombardment system, we introduced LIM18-GFP
into onion skin cells. Fluorescent microscopic observation did not show any particular intracellular distribution of LIM18-GFP signal as compared
with GFP alone (data not shown). However, using a transgenic BY-2 system we could observe differences in intracellular appearance of GFP-derived fluorescence. In addition, the result of cell fractionation experiments suggested the existence of LIM18-GFP in the nucleus. Although we could observe a relatively strong signal in the nucleus of the BY-2 cells expressing GFP alone, the result of cell fractionation experiment clearly show that the GFP is more abundant in the cytosol. On the other hand, the fluorescent signal derived from LIM18-GFP was more distinct in the nucleus than in the cytosol, which was confirmed by the cell fractionation experiment.

GFP is naturally dual-targeted to the cytosol and the nucleus, perhaps because its small size allows passive diffusion through the nuclear pore. Since
the molecular mass of LIM18-GFP (approx. 100-kDa) is much higher than the estimated molecular cut off range of the nuclear pore, we concluded that
the nuclear translocation of LIM18-GFP observed in this study is the result of active transport. The absence of nuclear localization signal and the presence of the EEDV signal suggest the intrinsic cytosolic localization of LIM18 (Hartl, 1996; Miernyk, 1997), but complexation with other nuclear targeted molecules may allow LIM18 molecules to be transported into the nucleus. This is probably the reason why LIM18−GFP is detected in the nucleus as well as in the cytosol. It will be interesting to know what kind of molecule interacts with LIM18 protein in BY-2 cells.

Previous studies on plant HSP70 revealed nuclear-localizing HSP70 immuno-reactive material in microspores of Brassica napus (Cordewener et al., 1995). To our knowledge, however, LIM18 is the first nuclear localizing meiosis specific HSP70-like molecule in plants. Nuclear translocation of LIM18 is particularly important when considering the mechanisms involved in the meiotic nuclear and/or chromosome organization. Further research for the identification of interacting molecules will be necessary for the elucidation of the function of LIM18. Identification of molecules that interact with LIM18 will provide important information for the understanding of the progression and regulation of meiosis in higher plants.

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