Characterization of cDNAs Induced in Meiotic Prophase in Lilium Microsporocytes

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Abstract

To identify and analyze genes functioning during reproductive cell formation in higher plants, cDNAs harboring the messages induced in meiotic prophase were isolated and characterized. A cDNA library constructed from microsporocytes in meiotic prophase of Lilium longiflorum was screened with a subtraction probe specific to meiotic prophase. Clones selected were classified into 18 groups by cross hybridization and partial sequencing. Northern blot analysis revealed that the transcripts corresponding to the respective cDNA groups began accumulating at the early stages of meiosis and exhibited clone-specific profiles during meiosis and the spore formation process. The amino acid sequences of the predicted gene products showed similarity with known gene products, e.g. heat shock proteins, serine proteases in Bacillus, and RAD 51 gene product in yeast. Half of the putative gene products had hydrophobic N-terminal regions, suggesting that they may function as signal peptides.

Key words: meiosis; zygotene; stage-specific genes; cDNA; Lilium longiflorum

1. Introduction

Meiosis is a complex process involving a highly regulated series of cytological and biochemical events, and the coordinated expression of a large number of genes. Characterization of such temporal and spatial gene expression has contributed toward a greater understanding of the mechanism of meiotic gene regulation during this process in lower eucaryotes. Especially in yeasts, mutants which express altered phenotypes at various stages of meiosis have been isolated and analyzed using both genetic and molecular biology techniques.1–3 In higher eucaryotes, on the other hand, such attempts to characterize those genes by the same strategy have been unsuccessful, due mainly to the difficulty of isolating mutants and the lack of appropriate analytical techniques.

Direct screening of gene libraries with differential probes has been a useful way to identify stage-specific genes for male gametogenesis in higher plants.4–6 A variety of genes specific to microsporogenesis,7,8 meiosis,9,10 mature pollens,11–16 and the tapetum17–20 have been isolated and characterized. To better understand the whole process, however, more genes which take part in this process need to be identified and analyzed. In this study, we isolated cDNA clones preferentially expressed during meiotic prophase of microsporogenesis in Lilium longiflorum, by means of a cDNA subtraction screening method.21 Using these cDNAs as probes, the corresponding genes were characterized in terms of duration of transcription and function of the putative products.

2. Materials and Methods

2.1. Plant Materials

Floral buds of L. longiflorum cv. Hinomoto were used for this study. Estimation of the stages of the microsporogenesis was based on the correlation between bud length and the determination of meiotic stages22 by cytological examination. Anthers were isolated from the dissected buds. Microsporocytes were extruded from the buds, pooled and washed with White’s medium to remove most of the contaminating tapetal nuclei, then stored at −20°C.

2.2. RNA Extraction

Total RNA was isolated according to the standard procedure using guanidium isothiocyanate and CsCl.23 Poly(A)+ RNA was purified from the total RNA by two rounds of oligo(dT) cellulose column chromatography.

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2.3. cDNA Library Construction

cDNA was synthesized from 5 μg of poly(A)+ RNA isolated from microsporocytes at the zygotene stage of meiotic prophase using a ZAP-cDNA synthesis kit (Stratagene). After synthesis of the second strand, an EcoRI adaptor was ligated to both cDNA termini, followed by an Xhol digestion to expose an Xhol site at the 3’ terminus of the strand. cDNA was purified by Sepharose CL-4B column chromatography and inserted into the EcoRI-Xhol site of lambda Zap (Stratagene) vector.

2.4. Subtraction Hybridization

A subtraction probe was prepared using a Subtracter I subtraction kit (Invitrogen). 32P-labeled cDNA was synthesized using 1 μg of poly(A)+ RNA from microsporocytes at the zygotene stage as a template and oligo(dT) as a primer. cDNA was then mixed with 10 μg of biotinylated poly(A)+ RNA from young anthers and incubated at 65°C for 48 h for hybrid formation. The hybrid molecules were removed from the mixture by binding with streptavidin followed by phenol extraction. This subtraction process was repeated again and the final single-stranded 32P-cDNA was used as a probe for screening of the cDNA library.

2.5. Northern Hybridization

Each lane was loaded with 1.5 μg of poly(A)+ RNA or 10 μg of total RNA which was then fractionated on 1% agarose gels containing 2.2 M formaldehyde. Blotting and hybridization were carried out as described previously.24

2.6. Amplification of the 5’ Terminal Region of mRNA

To analyze the primary structure of the region absent in plim3 clone, amplification of the 5’ terminal region of mRNA by a polymerase chain reaction (PCR) technique, known as 5’ RACE (rapid amplification of cDNA ends), was carried out. Two 20-mer oligonucleotides, NN7 (5’GAGAACTGCAATGGTCTCGG3’) and NN8 (5’ACCGAGCTACTCCATCAGAG3’), complementary to the 5’ terminal region of the plim3 cDNA were synthesized as primers. NN8 was mixed with 11 μg of total RNA from microsporocytes at zygotene, then cDNA was synthesized by primer extension reaction with reverse transcriptase. After the addition of a dG-tail at the 3’ terminus of the cDNA, the region between the binding site of NN8 and the dG-tail was amplified by PCR using NN8 and oligo(dC) as primers. The amplification product was subjected to a second round of PCR using NN7 and the oligo(dC) primers. The final product was purified and cloned into an Smal site in M13mp19 for sequencing analysis.

2.7. General Methods for Gene Cloning, DNA and RNA Manipulation, DNA Sequencing, and Database Search

Culture of Escherichia coli and phages, purification of plasmids and gene cloning were carried out according to the methods described by Sambrook et al.23 The sequences of the 5’ terminal regions of cDNAs were determined using double-stranded plasmid DNAs as templates with a T3 sequencing primer. Sequence determination of the entire cDNAs and primary structure analyses by referring to databases were carried out as described earlier.24

3. Results

3.1. Isolation of Meiotic Prophase-Specific cDNA Clones

cDNA clones harboring the genetic messages expressed preferentially during meiotic prophase were isolated by screening a cDNA library representing the miRNAs transcribed at the zygotene stage using a stage-specific probe. The probe was prepared by subtracting the messages expressed in the cells undergoing pre-meiotic vegetative growth from those expressed in microsporocytes at the zygotene stage, by subtraction hybridization.21 Approximately 1 x 106 plaques from the library were hybridized with the subtraction probe, and 500 clones which showed intense labeling signals were selected. These clones were subjected to cross hybridization, followed by partial sequencing of the 5’-terminal region of each cDNA, and were classified into 18 groups. A clone containing the longest insert was chosen from each group and used for further analyses. The original cDNA clones and the corresponding genes were termed plim and LIM (Lily messages Induced at Meiosis), respectively, as described in Table 1.

The transcripts of the 18 genes were visualized by Northern blotting and the lengths of the transcripts were estimated (Fig. 1). As shown in Table 1, most of the plim plasmids contained cDNA fragments which were more than 80% of the lengths of their corresponding transcripts.

3.2. Specificity of Gene Transcription

Transcription of the genes identified above were examined with respect to time specificity of expression during microsporogenesis as well as tissue specificity. poly(A)+ RNAs from several stages of microsporogenesis and the total RNAs from tissues including mature pollens, stems and leaves were separated on denaturing gels and Northern blotting was carried out using the cDNA portions of the plim plasmids as probes. LIM2 and LIM13 were not subjected to this analysis since the nucleotide sequences
Fig. 1. Northern blot analysis of LIM genes. Poly(A)^+ RNAs from meiocytes and total RNAs from other tissues were separated on denaturing agarose gels and Northern hybridization was carried out using corresponding plim cDNA inserts as probes. DNA fragments containing: H3, histone H3 genes in wheat; EF1, elongation factor 1a gene in lily (unpublished); rbc, large subunit of ribulose-bis-phosphocarboxylase gene in tobacco; and tub, α-tubulin gene in lily (unpublished) were used as control probes. Y, young anthers; I, interphase and leptotene stages; Z, zygotene stage; P, pachytene stage; D, diplotene-diakinesis stages; T, tetrads; M, total meiocytes; Po, mature pollens; S, stems; L, leaves.
Characterization of Genes Expressed during Meiosis

Table 1. Summary of LIM genes and the corresponding cDNA clones.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Transcript (bp)</th>
<th>Clone</th>
<th>cDNA insert length (bp)</th>
<th>Product (aa)</th>
<th>Accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIM1</td>
<td>500</td>
<td>plim1</td>
<td>472</td>
<td>90</td>
<td>D21807</td>
</tr>
<tr>
<td>LIM2</td>
<td>500</td>
<td>plim2</td>
<td>467</td>
<td>90 (95)</td>
<td>D21808</td>
</tr>
<tr>
<td>LIM3</td>
<td>500</td>
<td>plim3</td>
<td>263 (515)²</td>
<td>90 (95)</td>
<td>D21809</td>
</tr>
<tr>
<td>LIM4</td>
<td>500</td>
<td>plim4</td>
<td>490</td>
<td>100 (100)</td>
<td>D21810</td>
</tr>
<tr>
<td>LIM5</td>
<td>700</td>
<td>plim5</td>
<td>642</td>
<td>48 (135)</td>
<td>D21811</td>
</tr>
<tr>
<td>LIM6</td>
<td>550</td>
<td>plim6</td>
<td>547</td>
<td>113 (117)</td>
<td>D21812</td>
</tr>
<tr>
<td>LIM7</td>
<td>1050</td>
<td>plim7</td>
<td>1029</td>
<td>203 (219)</td>
<td>D21813</td>
</tr>
<tr>
<td>LIM8</td>
<td>1700</td>
<td>plim8</td>
<td>1647</td>
<td>513 (518)</td>
<td>D21814</td>
</tr>
<tr>
<td>LIM9</td>
<td>2500</td>
<td>plim9</td>
<td>2570</td>
<td>795 (813)</td>
<td>D21815</td>
</tr>
<tr>
<td>LIM10</td>
<td>700</td>
<td>plim10</td>
<td>694</td>
<td>155 (155)</td>
<td>D21816</td>
</tr>
<tr>
<td>LIM11</td>
<td>1000</td>
<td>plim11</td>
<td>841</td>
<td>198 (203)</td>
<td>D21817</td>
</tr>
<tr>
<td>LIM12</td>
<td>750</td>
<td>plim12</td>
<td>682</td>
<td>147 (153)</td>
<td>D21818</td>
</tr>
<tr>
<td>LIM13</td>
<td>700</td>
<td>plim13</td>
<td>545</td>
<td>48 (135)</td>
<td>D21819</td>
</tr>
<tr>
<td>LIM14</td>
<td>800</td>
<td>plim14</td>
<td>722</td>
<td>169 (170)</td>
<td>D21820</td>
</tr>
<tr>
<td>LIM15</td>
<td>1500</td>
<td>plim15</td>
<td>1395</td>
<td>349</td>
<td>D21821</td>
</tr>
<tr>
<td>LIM16</td>
<td>2100</td>
<td>plim16</td>
<td>2048</td>
<td>535 (580)</td>
<td>D21822</td>
</tr>
<tr>
<td>LIM17</td>
<td>2200</td>
<td>plim17</td>
<td>2156</td>
<td>443 (474)</td>
<td>D21823</td>
</tr>
<tr>
<td>LIM18</td>
<td>2200</td>
<td>plim18</td>
<td>2195</td>
<td>649 (651)</td>
<td>D21824</td>
</tr>
</tbody>
</table>

1) Lengths of the transcripts were estimated by Northern blot analysis. 2) Information obtained by 5′-RACE was compiled and shown in the parenthesis. 3) Numbers of amino acid residues were counted from the first methionine residue, and shown in the parenthesis. 4) Accession numbers in the GSDB, DDBJ, EMBL, and NCBI nucleotide sequence databases.

of the corresponding plim clones were so closely related to those of plim1 and plim5, respectively (see below).

As shown in Fig. 1A, most of the transcripts identified were first detected at the zygote stage of meiotic prophase and continued to be expressed throughout meiosis (LIM1, 3, 4, 5, 6, 7, 9, 10, 11, 14, and for LIM6, 8, 12, 16 with lower intensity). Transcription of LIM17 and LIM18, however, were detected at low levels even before entrance to meiosis and these transcripts accumulated at zygote followed by a gradual decrease toward the end of meiosis. LIM15, on the other hand, was shown to be transcribed only during early prophase.

None of the LIM transcripts which accumulated during meiosis were detected in mature pollen (Fig. 1B), indicating that rapid degradation of the transcripts took place during pollen maturation. The transcripts were observed in neither stems nor leaves except for LIM14, where a band of slightly shorter length was detected in stems.

3.3. Structural Features of the Putative Gene Products

To obtain information on the primary structure of the putative gene products of the 18 genes identified in this study, the entire nucleotide sequences of the cDNAs were determined by the dideoxy chain termination method. Figure 2 shows the structural features of the cDNAs. For LIM3, information on the 5′-terminal region of the mRNA was obtained by 5′-RACE. A distinct open reading frame (ORF) was identified in each clone. The ORFs in plim 1 and 15 were closed by an in-frame termination codon at their 5′ termini, indicating that the entire coding regions were included in the clones, while the ORFs in other clones were not. Comparing the lengths of the transcripts with those of corresponding cDNA sequences analyzed (Table 1), however, we have concluded that the majority of gene products was represented in the cDNA clones.

The nucleotide and amino acid sequences of the putative LIM gene products shown in Fig. 2 were compared with sequences in the nucleic acid and protein databases, respectively. The translated amino acid sequences were also subjected to a motif search. The results are summarized in Figs. 3 and 4. Notable features of the LIM genes and their products are as follows:

LIM10, 11 & 12 The putative gene products of LIM10, 11, and 12 showed sequence similarity to the conserved region of a small heat shock protein family, such as Hsp17.5 in Glycine max (Fig. 3A).²⁶ In the N-half of Lim11, a repeating 9-mer, P(S/T)X(S/T)YAIDA, was observed.

LIM18 LIM18 belongs to a member of a heat shock protein 70 (HSP70) gene family. The putative product of LIM18 is 72 kDa and has more than 85% similarity with Hsp70 in Petunia (Fig. 3B).²⁷ The
Fig. 2. Physical maps of the cDNAs in plim clones. Major open reading frames (ORFs), and 5' and 3' untranslated regions are indicated by open boxes and solid lines, respectively. Upstream regions of the first methionine codons are represented as ORFs unless 5'-terminus of the ORF is closed by an in-frame termination codon. The region which was amplified by 5'RACE is shown by a bar for plim3. The major restriction sites are indicated above the sequences by the following abbreviations: Ba, BamHI; Bg, BglII; H, HindIII; K, KpnI; P, PstI; Rfl, EcoRI; RV, EcoRV; Sa, SacI; Sm, Smal; Xb, XbaI; Xh, XhoI. The horizontal scale represents the cDNA length in kb. The nucleotide sequence data were deposited in the GSDB, DDBJ, EMBL, and NCBI databases under the accession numbers shown in Table 1.
Fig. 3. Comparison of the amino acid sequences of the putative LIM gene products with those of known gene products. The entire or the partial sequences of the putative products of the LIM genes are aligned with those of known gene products. The positions showing amino acid identity are indicated by shadowing. A: The entire sequences of the putative gene products of LIM10, LIM11, and LIM12 in Glycine max are aligned. The repeating unit in the LIM11 gene product is indicated by bars. B: The sequences of the LIM18 gene product downstream of the first methionine and the entire sequence of Hsp70 in Petunia are aligned. C: The entire sequences of the gene products of LIM15, DMC1/ISC1, and RAD51 are aligned. The consensus sequences for the nucleotide binding motif are indicated by underlining. D: The amino acid sequences of three conserved regions (a, b, and c) between the LIM3 gene product and subtilisin in Bacillus subtilis are aligned. E: The sequences of the putative gene product of LIM1, LIM2, LIM3, and fill in Antirrhinum majus are aligned. For the LIM8 and LIM9 products, only the sequences after the first methionine are shown. F: The entire sequences of the putative gene products of LIM5, LIM6, and LIM13 are aligned. The positions showing amino acid identity with the LIM13 product are indicated by shadowing. G: The entire sequence of the putative gene product of LIM14 is shown. The positions of glycine residues in the repeating structure are indicated by shadowing.
Fig. 3. Continued
Fig. 4. Sequence features of the predicted LIM gene products. The regions, downstream of the first methionine residues for Lim1, Lim2, Lim3, Lim15, and Lim18, and downstream of the first amino acid residues corresponding to the 5' termini of the ORFs for the rest of the genes, were subjected to analyses. The horizontal scale represents numbers of amino acid residues from the N-terminus. Occurrence of hydrophobic amino acid residues was indicated by blue vertical bars (long: aromatic, short: aliphatic) and hydrophilic residues by either green (Arg, Lys and His: long, medium and short upward bars; Glu and Asp: long and short downward bars) or red vertical bars (long: amides, short: alcohols). Pro was shown by black bars and the positions of Ala, Gly and Cys were left unmarked. Above the lines of amino acid residues, the regions that showed similarities to known genes were indicated by horizontal lines with arrowheads at both ends, location of protein motifs that matched those in the PROSITE motif database by black blocks, and significant hydrophobic regions by red blocks. Abbreviations are as follows: Am, Antirrhinum majus; Gm, Glycine max; Sc, Saccharomyces cerevisiae; P, Petunia.
LIM15 LIM15 appears to code for a 38-kDa polypeptide, consisting of 349 amino acid residues. As shown in Fig. 3C, the translated amino acid sequence of LIM15 showed a high degree of similarity to those of Rad51, a gene product which is involved in DNA repair and homologous recombination in *Saccharomyces cerevisiae*, and its homologues in fungi, 32–34 chicken, 35 and mammals. 34,36,37 Similarity was also found to that of Dmc1/Isc2, a gene product which is involved in meiosis and meiotic homologous recombination in *S. cerevisiae*. 24,38 The similarity among these polypeptides extends over almost the entire length except for the N-terminal region. Amino acid similarity of LIM15 with Rad51 homologue of human and Dmc1/Isc2 is 52% and 55%, respectively. Limited sequence similarity was also found between LIM15 and Rad 57, 39 particularly in the regions around the nucleotide binding domains (Fig. 3 in ref. 24).

LIM9 Portions of the putative product of LIM9 showed limited sequence similarity to serine proteases such as subtilisin in *Bacillus subtilis*. 40 Three amino acid residues, aspartic acid, histidine and serine, indispensable for high serine protease activity, are conserved in these portions (Fig. 3D).

LIM1, 2 & 3 plim1 has a closed ORF which codes for a 9-kDa polypeptide. The product has a putative signal peptide and shows similarity to a product of the *fill* gene, a stamen-specific gene in *Antirrhinum majus*, 41 including 42% amino acid identity (Fig. 3E). The translated amino acid sequence of Lim2 is identical to that of Lim1 except for one residue. The transcript of the *LIM1-LIM2* family may constitute one of the most abundant class of messages induced in meiotic prophase since approximately 80% of the cDNA clones selected by subtraction screening hybridized to the *LIM1* probe. The putative LIM3 product showed a high degree of similarity to that of Lim1, and to the *fill* product with 48% amino acid identity (Fig. 3E).

LIM5, 6 & 13 The putative products of LIM5, 6 and 13 did not show any similarity to known sequences. The translated amino acid sequence of Lim5 is 98% identical to that of Lim13 and is similar to Lim6 to a lesser extent (Fig. 3F). Hydrophobic N-terminal regions were observed in all three.

LIM14 A characteristic feature of the putative product of LIM14 is a hydrophobic N-terminal region followed by a glycine- and serine-rich repeating structure in which glycine appears every other residue (Fig. 3G). Structural proteins such as glycine-rich cell wall proteins in plants, and cytokeratin in mammals are known to have a similar glycine-rich structure. 42,43

LIM4, 7, 8, 16 & 17 Sequence features of the translated amino acid sequences of the ORFs found in plim4, 7, 8, 16, and 17 are shown in Fig. 4. Comparison of these sequences with a protein database did not show any sequence similarities with known gene products.

4. Discussion

In this study, we have identified 18 genes of which transcription is induced during early prophase of meiosis in microsporogenesis of *L. longiflorum*. Most portions of the coding regions of the genes were revealed by the structural analysis of the corresponding cDNAs, and possible function of the products was predicted by comparing the translated amino acid sequences with those of polypeptides that have already been characterized.

Expression of heat-shock genes during reproductive cell formation has been reported in *Drosophila*. 44 *Saccharomyces*, 45 and *Lilium* 9, 10, although their significance in this process is not known. In *Lilium*, transcription of a gene that shows homology to a small heat shock protein gene in meiotic prophase has been reported. 10 The translated amino acid sequence of the product of this gene is almost identical to that of *LIM10*, suggesting that they constitute a family of small heat shock protein genes in *Lilium*.

LIM9 is presumed to code for a serine protease. Sequence similarity to the known proteases is observed in three regions along the polypeptide, on which three residues are located which are highly conserved among serine proteases (Fig. 3D); however, the distance between conserved histidine and serine was longer than those of other proteases, as was reported in Vpr, an extracellular protease in *Bacillus subtilis*. 46 A hydrophobic N-terminus of the putative product also suggests an extracellular role for the LIM9 product.

The putative product of LIM15 shows significant sequence similarity to *RAD51* and to *RAD57* to a lesser extent. Both of the products of *RAD51* and *RAD57* are known to be involved in DNA repair and homologous recombination in *S. cerevisiae*. 29–31,39 Considering that the transcription of LIM15 occurs only in early meiotic prophase, it is plausible that Lim15 takes part in DNA metabolism in meiotic recombination. In our recent study, 24 it was shown that disruption of *ISC2*, a homologous gene of LIM15 in *S. cerevisiae*, arrested the reproductive cell cycles at meiotic prophase. Bishop et al. performed an intensive study of this gene, *DMC1* according to their designation, 38 in which they have shown...
that the mutation on the gene caused loss of reciprocal recombination and of synaptonemal complex formation during meiosis.

fil1 is a gene expressed in the filament of the stamen and at the bases of the petals during flower development in the wild type, yet not in the homeotic mutant, of *A. majus*. The putative products of *LIM1, 2 and 3* show sequence similarity to that of the *fil1* product, including hydrophobic N-termini. The transcripts of these *LIM* genes accumulate abundantly during late meiosporegenesis (Fig. 1). These characteristics suggest that *LIM1, 2 and 3*, as well as *fill*, code for polypeptides which function as extracellular structural components. The putative products of *LIM5, 6 and 13* also share the same characteristics.

Despite of tractability of *Lilium* for biochemical studies on meiosporegenesis, the absence of genetic studies limits further analysis of the functional aspects of the *LIM* genes. To overcome this obstacle, isolation of homologous genes and subsequent characterization using systems which permit the use of the appropriate techniques for genetic studies seem to be promising. Using pUM15 cDNA as a probe, we have isolated a homologous gene of *LIM5* from the genomic library of *S. cerevisiae*. This gene was shown to be essential to the progression of meiosis by a functional analysis using reverse genetic techniques. We have also isolated similar sequences from the genomes of *Schizosaccharomyces pombe* (manuscript in preparation) and plants (unpublished). Further screening efforts would undoubtedly lead to the isolation and identification of homologues with other *LIM* genes, and would help clarify the possible function of such genes during meiospore formation.

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