Original Paper Plant Biotechnology, **18** (2), 151-156 (2001)

Isolation of a Promoter that Directs Microsporogenesis-Associated Gene Expression in *Lilium longiflorum*

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Received 27 December 2000; accepted 20 February 2001

Abstract

LIM genes, identified as cDNAs from pollen mother cells of *Lillium longiflorum*, are specifically induced at meiosis I of microsporogenesis. *LIM8* encodes a protein which is highly homologous to amino acid transporters and exhibits high expression levels at late stages of microsporogenesis. To study the regulated expression of microsporogenesis-associated genes, we isolated a genomic clone containing the 5' upstream region of *LIM8* gene by a PCR-based approach. Sequence analysis of the 0.6 kb genomic DNA fragment revealed the presence of putative transcription factor binding sites. However, no sequence similarity was found in promoter regions of *LIM8* gene and previously described meiosis-associated genes. Transient assay by microprojectile bombardment demonstrated that the putative promoter region of *LIM8* gene is able to direct tissue-specific expression of the luciferase reporter gene in lily microsporocytes.

Introduction

Microsporogenesis is a highly organized indispensable event for the sexual reproduction of higher plants. It is a series of processes including organ development, cell differentiation and meiosis. Although recent advances in research on mammals have unveiled the mechanisms of meiosis and spermatogenesis, much less is known about meiosis and microsporogenesis in higher plants. Lily has been used to study meiosis because of the accessibility of the male gametophyte, the synchronous development among sporogenous cells within an anther, and the large size of their cells and chromosomes (Erickson, 1948; Hotta *et al.*, 1985). By using the subtractive hybridization screening technique, Kobayashi *et al.* (1994) isolated cDNA clones which are specifically induced at the meiotic prophase I of microsporogenesis in *Lilium longiflorum*. Isolated cDNA clones were classified into 18 groups and the corresponding genes were named *LIM* (Lily messages Induced at Meiosis). We have characterized a number of *LIM* gene products. However, the regulatory mechanism involved in *LIM* gene expression is not necessarily clear (Mousavi *et al.*, 1999; Ogata *et al.*, 1999; Minami *et al.*, 2000).

The purpose of this study is to characterize the microsporogenesis-associated gene expression by isolating the genomic DNA fragment involved in the regulated expression of *LIM* genes. The *LIM8* gene is induced at the zygotene stage of microsporogenesis and encodes a putative amino acid transporter (Uefuji *et al.* unpublished). Because of its specific expression pattern during microsporogenesis, we have undertaken a study of the regulated expression of *LIM8* gene for the elucidation of mechanisms of microsporogenesis-associated gene expression in higher plants.

Here we report the isolation of the *LIM8* promoter sequence from the *L. longiflorum* genomic DNA and partial characterization of the promoter activity that directs microsporogenesis-associated gene expression.

Materials and Methods

Plant materials

Flower buds of lily (*Lilium longiflorum* cv. Hinomoto) were categorized according to their length, which was calculated from the base of the pedicel to the tip of the sepals. Anthers were dissected from buds, soaked in liquid nitrogen and stored at -80 ℃ . The developing microspores of each stage were isolated from the corresponding

flower buds, pooled and washed with White's medium to remove the sporophytic contamination, then stored at -80 ℃ as described previously (Mousavi *et al.*, 1999).

RT-PCR analysis

Total RNA was extracted from various tissues and bud stages using an RNeasy plant mini kit (Qiagen). The RT-PCR reaction was performed with an RNA PCR kit (TaKaRa) using the following primers:

LIM8F (5'-AATGCAATTGGGATCATCGC-3');
LIM8R (5'-GCTTATATTCACACCAGTCTCG-ACC-3');
LiEF1-1 (5'-GAGGCAGACTGTTGCTGTCGG-3') and
LiEF1-2 (5'-AGCAGACTGAAATGAAGATGC-3').

The PCR reaction mixture was kept at 94 ℃ for 1 min., followed by 25 cycles at 94 ℃ for 30 sec., 57 ℃ for 30 sec., 72 ℃ for 1 min. Amplified products were separated on a 1.0 % agarose gel and stained with ethidium bromide.

PCR amplification of lily genomic DNA

Zygotene stage microsporocytes (1.5 m*l*), washed with White's medium, were ground to a powder in liquid nitrogen in a mortar with a pestle. Extraction and purification of lily genomic DNA was carried out according to published methods (Murray and Thompson, 1980). The *LIM8* promoter region was amplified by inverse PCR (Ochman *et al.*, 1988) using the LA PCR *in vitro* Cloning Kit (TaKaRa). The self-ligated genomic DNA, following *Hae*II digestion, was used as template for the first-round PCR with LIM8F1 primer (5'-ATTGTGAGCAT-GAGAGGCTATCTGG-3') and LIM8R1 primer (5'-AGTGGTGATCGGGTTAGGGATG-3'). The PCR reaction mixture was kept at 94 ℃ for 1 min., followed by 30 cycles at 98 ℃ for 20 sec., 64 ℃ for 30 sec., 68 ℃ for 2 min. For the nested PCR, the first-round PCR products were further amplified with LIM8F2 primer (5'-CCATACTCTCAGTTCTGG-CATTG-3') and LIM8R2 primer (5'-GTCAC-CGGCATGGACTGT-3') by 30 cycles at 98 ℃ for 20 sec., 58 ℃ for 30 sec., 68 ℃ for 2 min. after the reaction mixture was kept at 94 ℃ for 1 min. An amplified DNA sample was separated on a 1.0 % agarose gel and the band was cloned into pT7blue T-vector (Novagen). DNA sequencing was carried out using a Perkin Elmer Sequencing kit according to the manufacturer's instructions by cycle sequencing with dye-labeled M13 reverse and -21 M13 primers on an automated DNA sequencer (ABI 373 Stretch, Applied Biosystems).

To prepare the promoter DNA in a different length, the following primers were designed: LIM8F3 (5'-CGCTTACTAGGAGACAACCTAG-CA-3'), LIM8F4(5'-GGCATGTGATTCTTTGC-TATT-3'), LIM8F5 (5'-CCCAAAGTACCTAGG-ACTTA-3'), LIM8F6 (5'-GGTAAGAGTGCCA-TTGTTGG-3') and LIM8F7 (5'-AAGCTTAGAA-TGACCCACCACT-3'). For the amplification of lily genomic DNA with these primers, the PCR reaction mixture was kept at 94 ℃ for 1 min., followed by 30 cycles at 98 ℃ for 20 sec., 58 ℃ for 30 sec., 68 ℃ for 1 min. An amplified DNA sample was separated on agarose gel and the isolated band was cloned into pT7blue T-vector.

Plasmid construction

LIM8 genomic fragments, 0.6 kb and 0.35 kb, containing the promoter region and partial coding sequence (see **Fig. 2B**) were excised by *Pst*I-*Bam*HI and *Hin*dIII-*Bam*HI from pT7blue T-vector and then blunted using a DNA blunting kit (TaKaRa). The genomic fragments were inserted into the *Hin*dIII (blunt)-*Nco*I (blunt) site of pBI221-luc+ to make pLIM8 (600)-luc+ and pLIM8 (350)-luc+ (see **Fig. 3A**; Matsuo *et al.*, 2001).

Transient assay by microprojectile bombardment

Microsporocytes at the leptotene and zygotene stages of lily were prepared and maintained as described previously (Tabata *et al.*, 1993). Samples were bombarded with 1.6 μm gold particles coated with 0.5 μg of each plasmid construct using a model PDS-1000/He particle delivery system (BioRad). After incubation at room temperature in the dark for 24 hours, promoter activities were measured by dual luciferase assay system (Promega) as described previously (Matsuo *et al.*, 2001).

Results

Expression of LIM8 mRNA

The specific expression pattern of *LIM8* gene during microsporogenesis was previously suggested by RNA gel blot analysis (Kobayashi *et al.*, 1994). However, the *LIM8* mRNA accumulation has not been studied any further. In order to obtain a detailed profile of *LIM8* gene expression, we conducted RT-PCR analysis using various plant tissues in addition to different stages of microsporogenesis and pollen development.

LIM8 was found to be expressed during microsporogenesis, but in contrast to the result obtained by RNA gel blot analysis (Kobayashi *et al.*, 1994), *LIM8* mRNA accumulation levels were increased from the zygotene to tetrad stages and remained at low levels in uninucleate pollen grains (**Fig. 1**, lanes

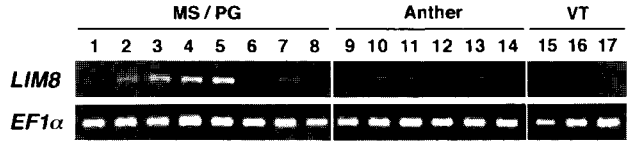


Fig. 1 Expression pattern of *LIM8* transcripts in Lily. Total RNA samples isolated from microsporocytes (MS), pollen grains (PG), anther and various vegetative tissues (VT) were subjected to RT-PCR analysis. MS, PG and anther samples were divided into different stages of microsporogenesis and pollen development: leptotene (lanes 1 and 9); zygotene (lanes 2 and 10); pachytene (lanes 3 and 11); diplotene- 2nd meiosis (lanes 4 and 12); tetrads (lanes 5 and 13); early uninucleate pollen grain (lanes 6 and 14); late uninucleate pollen grain (lane 7); binucleate pollen grain (lane 8). Vegetative tissues were from leaf (lane 15), stem (lane 16) and root (lane 17). *EF1a* was used as a control for all samples.

2 to **7**). On the other hand, no *LIM8* mRNA was detected in the samples from binucleate pollen grains, leaf, stem and roots (**Fig. 1**, lanes **8** and **15** to **17**). *LIM8* mRNA levels were higher in the samples from microsporocytes and pollen grains than from whole anthers at the same stage.

Isolation of the LIM8 promoter by PCR

The ligation product of *Hae*II digested lily genomic DNA was used as inverse-PCR template. Using LIM8F1 and LIM8R1 primers, we conducted the first round PCR. As a result of the nested PCR with LIM8F2 and LIM8R2 primers we obtained a 1.1 kb band.

Based on the sequence information of the 1.1 kb fragment, we designed the five primers LIM8F3, LIM8F4, LIM8F5 , LIM8F6 and LIM8F7 as described in Materials and Methods, for the amplification of lily genomic DNA with LIM8R2 (**Fig. 2A**). PCR with LIM8F6 and LIM8R2 primers and with LIM8F7 and LIMR2 primers resulted in the amplification of 600 and 350 bp DNA fragments, respectively. However, no DNA amplification was detected by LIM8F3, LIM8F4 and LIM8F5 primers (**Fig. 2A**). This suggests that the initial amplified product obtained by inverse PCR was the result of ligation of discontinuous genomic DNA fragments. After confirming the direct amplification from the genomic DNA, a 0.6 kb lily genomic DNA fragment containing the putative *LIM8* promoter region was obtained (**Fig. 2B**).

Examination of the *LIM8* genomic DNA sequence revealed the presence of putative transcription factor binding sites (**Fig. 2B**). However, sequence comparison with the 5'-upstream region of meiosis-associated genes, such as Arabidopsis *DMC1/LIM15* (Sato *et al.*, 1995) and lily *meiotin-1* (Hasenkampf *et al.*, 2000), revealed no conserved regulatory elements within the putative promoter region of these genes.

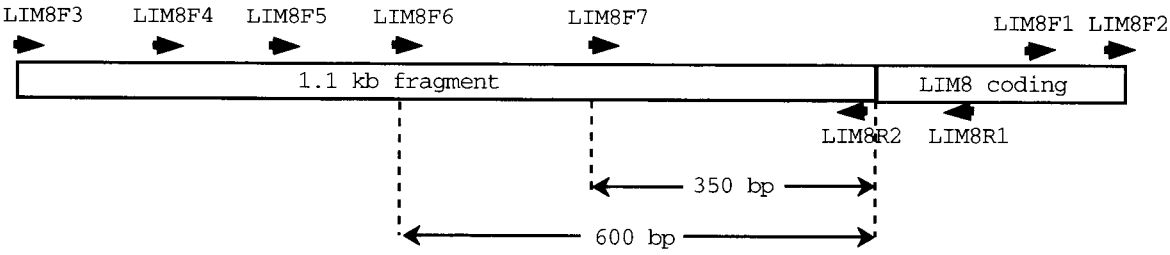
Tissue specific expression of LIM8 promoter

In order to examine the microsporogenesis-associated promoter activity, we fused the *LIM8* genomic DNA fragments to the firefly luciferase (F-luc) reporter gene and introduced them into lily samples by microprojectile bombardment. The cauliflower mosaic virus 35S promoter fused to the *Renilla* luciferase (R-luc) gene was used as a co-reporter to F-luc (**Fig. 3A**). Transient assay of the *LIM8* (600) promoter resulted in a relatively low activity in leaf: 0.4 % of the *35S* promoter. On the other hand, the *LIM8* (600) promoter exhibited higher activities in the whole anther containing microsporocytes and the isolated microsporocytes: 2.6 % and 14 % of the *35S* promoter, respectively (**Fig. 3 B** and **C**). A transient assay with the *LIM8* (350) promoter, a shorter version of the *LIM8* (600) promoter (**Fig. 3A**), resulted in a tissue-specific expression pattern similar to the *LIM8* (600) promoter with reduced expression levels (**Fig. 3 B** and **C**). A promoter-less luciferase gene showed F-luc activity indistinguishable from the background levels in all samples (data not shown). These results suggest that the *LIM8* genomic sequence obtained in this study is able to direct tissue and cell-type specific expression in lily.

Discussion

Pollen- and/or microspore-specific gene expression has been studied extensively and many specific promoters have been isolated and characterized (Twell, 1994). However, the promoter fragment that directs specific expression at early stages of microsporogenesis has not been identified so far. Recently, Hasenkampf *et al.* (2000) reported the isolation of a lily genomic DNA fragment of *meiotin-1* gene encoding a meiosis-specific histone H1-like protein. However, the promoter activity of *meiotin-1* genomic DNA has not been investigated. The promoter fragment of a meiosis-specific *RecA*-like gene, *DMC1*, has been identified and characterized in Arabidopsis (Klimyuk and Jones, 1997). Although the T-DNA insertion in *AtDMC1* resulted in a phenotype restricted to pollen fertility (Couteau *et al.*, 1999), the tissue specificity of Arabidopsis *DMC1* gene is not tightly regulated and relatively high levels of the gene expression were also observed in roots and tissue-cultured

(A)



(B)

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                                        LIM8 (600)
-601                                    GGTAAGAGTGCCATTGTTGGGACCTTATATACTAATATAGA
-560    AGAGGTTGTAACAAAGAATAATAAAATCAAAACACAGGATCAGACAGGAAATGATTCAATTGGGACGGTA
-490    TCATGCCATGCTGTAGGACGTCAGCATAGAATGAAAATGGACGAAAACTCATCAGCCCACGGTATCTTTA
-420    TAGTCATATAGGTAATAGCTGATATATTTAAACTATAAGTCAAGTCAGCCAAAGTCAGCTATGTTCAGAC
                LIM8 (350)
-350    CCAACTAAAGCTTAGAATGACCCACCACTAACCTACCCTTTCCCCCCAAAAGAAAATCTCACCAACCCTA
-280    ATTAACATGTGTTAATTGGACGGTAAATTAACTTAAACAATGCTAGGGCATTATAATCCCCAGAGCTAGA
-210    CGGTAGTGGGCGACAGTCCTTGCGCTTTAAAAAGTTTACCCTGTATTTTACCAGTTCCGTCACGGTTGGT
-140    GACGTGGCGAGTTACACCTGATCCGAGCCCACAAAGCATGACACACCAACCCCAGTCCTTCCCTCCTCAG
 -70    CTCCCAGCCTACATAAAGGGTTGGGCTGAGGGAGGGAGAGAAGCAAGCTGAAAGAGCCAGCCACCAAGCA
  +1    ATGGAAGTACAGTCCATGCCGGTGAC
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Fig. 2 PCR primer binding sites and the sequence of the *LIM8* genomic DNA isolated in this study.

(A) Schematic diagram of the DNA fragment and binding sites for primers used in this study. The amplified genomic DNA region corresponding to 600 bp and 350 bp fragments are indicated. **(B)** The sequence of the *LIM8* genomic DNA. The coding sequence is represented in italic letters, and the translation start codon in bold letters. The putative TATA-box and CAAT-box are boxed. The ASF-1 binding motif (TGACG: Lam *et al.*, 1989) and C-box (GACGTC: Foster *et al.*, 1994) are underlined. The endpoints of the *LIM8* promoters used for the transient assay are indicated above the sequence with arrows. The nucleotide sequence data of *LIM8* genomic DNA are available in DDBJ, EMBL and GenBank nucleotide sequence databases as accession number AB050987.

cells (Doutriaux *et al.*, 1998).

LIM cDNAs were originally identified by subtractive hybridization and the RNA gel blot analysis revealed that *LIM* genes are specifically expressed during microsporogenesis in *L. longiflorum* (Kobayashi *et al.*, 1994). As shown in **Fig. 1**, we confirmed the tissue specificity of *LIM8* mRNA accumulation by RT-PCR analysis. Unlike the Arabidopsis *DMC1*, it is clear that the *LIM8* gene expression in somatic tissue is below the detectable level. These results led to the suggestion to isolate the *LIM8* gene promoter and study the regulation of *LIM8* gene transcription.

In order to analyze transcriptional regulation, it is necessary to isolate the genomic DNA containing the 5' upstream region of the gene. However, because of its large genome size, we considered it difficult to isolate *L. longiflorum* genomic DNA fragment by any conventional method such as phage library screening. In this study, we exploited a PCR-based method and successfully cloned the genomic DNA fragment containing the putative promoter

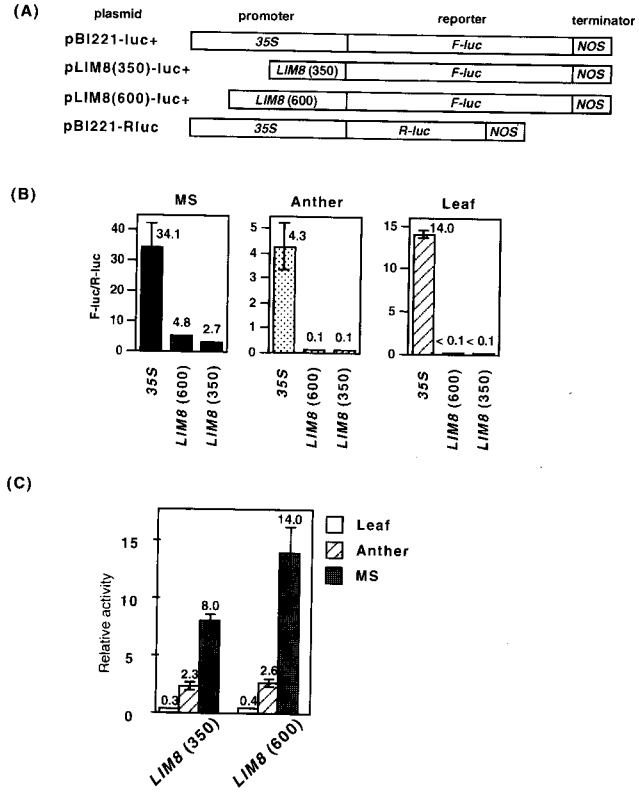

Fig. 3 Transient expression analysis of *LIM8* promoter in lily.

(**A**) Diagrams of the reporter fusion genes used for microprojectile bombardment. *LIM8* promoter constructs are translational fusion with F-luc coding sequence (for detailed information, see materials and methods). (**B**) Test plasmids, pBI221-luc+, pLIM8(600)-luc+ and pLIM8(350)-luc+, were co-bombarded with the reference plasmid pBI221-Rluc into microsporocytes (MS), anther and leaf and assayed for F-luc and R-luc activities by dual-luciferase assay. Activity, given as a ratio of F-luc/R-luc units, is averaged from three independent experiments. (**C**) Relative activity of the *LIM8* promoters. Relative activity is given as percentage of the CaMV35S promoter activity in each sample. Error bars show the S.D.

region of the *LIM8* gene. Since the procedure used in this study is simple and versatile, it would be particularly useful for the isolation of the promoter sequences of various plant species whose genome size is large and/or genomic sequence information is not readily available.

Sequence comparison of the promoter region of *LIM8* gene with previously described meiosis-associated genes such as *AtDMC1* and *meiotin-1* revealed no consensus sequence motif among these genes. On the other hand, as shown in **Fig. 2A**, the presence of transcription factor binding sites commonly found within the promoters of various plant genes suggests that the regulatory mechanisms involved in *LIM8* transcriptional regulation are, at least in part, shared by other plant promoters. In fact, the *LIM8* (350) fragment, lacking the ASF-binding motif (Lam *et al.*, 1989) and the C-box (Foster *et al.*, 1994) both of which function as positive regulatory elements in somatic tissues, exhibited reduced activities in the transient expression assay (**Fig. 3**).

The results obtained by a transient assay shown in **Fig. 3** demonstrated the ability of the *LIM8* promoter to direct tissue-specific expression. This is in good agreement with the expression pattern of *LIM8* mRNA shown in **Fig. 1**. The fact that the *LIM8* promoter exhibited very low activity in leaf tissue may indicate a specific repression of the promoter activity. Conversely, the relatively high expression level in meiotic microsporocytes suggests a possible involvement of the specific activation of *LIM8* gene transcription in microsporocytes. Because of its versatility, we exploited a transient assay system by microprojectile bombardment in this study. Therefore, the evidence for promoter specificity that we could obtain was the activity measured only by a transient assay with reporter genes. For the evaluation of accurate promoter activity, however, it is important to analyze microsporogenesis-associated gene expression in transgenic plants. The use of stable transformants, as opposed to the extra-chromosomal expression by a transient assay, is particularly important when considering the unique chromosome behavior and nuclear architecture during the process of meiotic progression. Because generating the transgenic lily is technically difficult, we are currently testing the *LIM8* promoter activity and specificity using transgenic tobacco and Arabidopsis.

Acknowledgments

This work was supported by the Japanese Society for the Promotion of Science Research for the Future Program JSPS-RFTF96R16002 and by a Grant-in-Aid for Scientific Research on Priority Areas from the Ministry of Education, Science and Culture of Japan (No. 12025222).

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