

## Identification of Novel Microsporogenesis-Associated Genes Encoding Proteins with a Nuclear Localization Signal.

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### Abstract

Microsporogenesis is a highly organized indispensable event for the sexual reproduction of higher plants. The process includes a series of organ development, cell differentiation and meiosis. We examined the functional genes involved in microsporogenesis by random sequencing using a cDNA library from lily zygotene stage microsporocytes and isolated two cDNAs encoding novel gene products. Deduced amino acid sequences of both gene products, designated *M355* and *M404* contained a cluster of basic amino acid residues that may constitute a nuclear localization signal. RT-PCR analysis indicated that the temporal and spatial expression of *M355* and *M404* is associated with early stages of microsporogenesis or meiosis. Transient expression of GFP fusion proteins in onion epidermal cells revealed nuclear localization activity of both proteins. These results suggest that *M355* and *M404* are involved in a nuclear event during the progression of microsporogenesis in lily.

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**Key words:** meiosis, microsporogenesis, nuclear localization signal.

### Abbreviations

GFP, green fluorescent protein; NLS, nuclear localization signal; PMC, pollen mother cell; RACE, rapid amplification of cDNA ends; RT-PCR, reverse transcription-polymerase chain reaction.

### Introduction

Microsporogenesis begins with the division of a diploid sporophytic cell, giving rise to the tapetal initial and the sporogenous initial (pollen mother cell). The sporogenous cell undergoes meiosis, giving rise to a tetrad of haploid cells, that are released as free microspores by the action of enzymes produced by the tapetum layer of the anther. These uninucleate microspores undergo an asymmetric mitotic division, resulting in a pollen grain containing two cells, a larger vegetative cell and a smaller generative cell that is enclosed within the vegetative cell and finishes when the pollen grain is released from the anther. All these processes in-

volve a complex program of spatially and temporally regulated genes in the anther. Although major cytological and biochemical events that accompany microsporogenesis have been well studied, relatively little information is available concerning the regulation of such events at the molecular level.

Recently, using an Arabidopsis system, the genes involved in microsporogenesis have been identified. Young *et al.* reported the *SPOROCTELESS (SPL)* gene, also known as the *NOZZLE* gene (Schiefthaler *et al.*, 1999), that is essential for sporogenesis in both male and female organs in Arabidopsis (Young *et al.*, 1999). The *spl* mutation blocks the differentiation of primary sporogenous cells into microsporocytes and anther wall formation and the *SPL* gene encodes a novel nuclear protein related to MADS box transcription factor. From these findings, Young *et al.* (1999) suggested that the development of anther walls and the tapetum and microsporocyte formation are tightly coupled and that the *SPL* gene product likely functions as a transcriptional regulator essential for microsporogenesis.

One of the most effective approaches for under-

standing the molecular mechanisms involved in microsporogenesis is to isolate and characterize genes that are expressed specifically in meiocytes. Previously, 18 meiosis-associated cDNAs were isolated from microsporocytes of a monocotyledonous plant lily (*Lilium longiflorum*) and their corresponding genes were designated as *LIM* genes (Kobayashi *et al.*, 1994). Among the gene products, LIM15 that takes part in meiosis-specific homologous chromosome recombination has been well characterized (Kobayashi *et al.*, 1993; Terasawa *et al.*, 1995). However, genes that are expressed in somatic cells also play an important role in meiotic progression. For example, a meiotic recombination checkpoint was recently reported to be controlled by mitotic checkpoint genes in yeast (Lydall *et al.*, 1996). In addition to studying the specific genes, it is also important to study genes expressed in somatic cells for elucidating the processes involved in microsporogenesis.

Random cDNA sequencing can be used to isolate many functional genes expressed in a particular tissue or under a specific environmental condition. Recently, a large number of cDNA clones were identified by random sequencing from plants including maize (Keith *et al.*, 1993), *Arabidopsis thaliana* (Höfte *et al.*, 1993; Newman *et al.*, 1994), *Brassica napus* (Park *et al.*, 1993) and rice (Uchi-miya *et al.*, 1992; Sasaki *et al.*, 1994; Hihara *et al.*, 1997).

In order to identify genes expressed at microsporogenesis, we carried out random sequencing using a lily cDNA library made from zygotene stage microsporocytes. To avoid redundancy of cDNA clones and to obtain novel sequence information efficiently, a simple and versatile "self-hybridization" strategy was developed and single-run DNA sequence data were obtained from 418 cDNA clones. Deduced amino acid sequences of 171 cDNA clones, which comprised 41% of the selected cDNA clones, showed significant similarity to amino acid sequences registered in the database. These included cDNA sequences for previously identified meiosis-associated genes. However, 59% of the selected clones showed no significant homology to known gene products. Interestingly, compared with the previously described random sequencing projects, the ratio of cDNAs encoding novel proteins was relatively high and some of the predicted amino acid sequences encoded by the cDNAs exhibited interesting features (Morohashi *et al.* 2000).

Here we report novel meiosis-associated genes, designated *M355* and *M404*, encoding proteins with putative nuclear localization signals. The organ- and tissue-specific expression of both genes indi-

cated that the temporal and spatial expression of *M355* and *M404* are associated with early stages of microsporogenesis. The intracellular targeting of the fusion protein using a GFP tag system is also reported.

## Materials and Methods

### *Plant material*

*Lilium longiflorum* cv. Hinomoto was used in this study. Flower buds were categorized according to their length and the developmental stage was determined cytologically as described previously (Erickson, 1948). Anthers were dissected from buds, frozen in liquid nitrogen and kept at  $-80^{\circ}\text{C}$ . Developing microsporocytes were extruded from corresponding buds, pooled and washed with White's medium to remove sporophytic contamination and kept at  $-80^{\circ}\text{C}$ .

### *5' RACE and RT-PCR*

Total RNA was isolated from various tissues and anthers of corresponding stages by using an RNeasy kit (QIAGEN), and treated with RNase free DNase. 5' RACE was carried out with 1.0  $\mu\text{g}$  of zygotene to pachytene stage anther total RNA by using a SMART RACE cDNA amplification kit (Clontech) with primers M355R (5'-AACACATCGGT-CATCT GTGC-3'), M404R (5'-GCATTC-CCCGCATAA CACCAACA-3') and M404R2 (5'-CAGCCTGCATCTGCATCACCTGT-3').

The RT-PCR was conducted using an RNA-PCR kit (Takara). The first strand cDNA was synthesized from 500 ng of total RNA and PCR was carried out with the following primers: M355F (5'-AGAGATGGACTCTGCTGCTAGG-3'), M355R, M404F (5'-GTGGAGGATGAGAGGAGGGAGA-3'), M404R, EF1aF (5'-GAGGCAGACTGTT-GCTGTCGG-3') and EF1aR (5'-AGCAGAC-TGAA ATGAAGATGC-3'). PCR conditions were  $94^{\circ}\text{C}$  30 s,  $55^{\circ}\text{C}$  30 s and  $72^{\circ}\text{C}$  1 min, 20 cycles. PCR products were separated on 1.2% agarose gel, and then transferred to a Hybond-N+ membrane (Amersham). Labeling of cDNAs and hybridization were carried out according to the manufacturer's protocol (Gene Images AlkPhos Direct labeling and hybridization system, Amersham) and detected with chemiluminescent detection reagent (CDP-Star<sup>TM</sup> detection reagent, Amersham).

### *Subcellular localization of GFP fusion proteins*

A GFP expression vector, designated 221-EGFP-C1, was constructed by inserting the *Bam*HI-*Nhe*I fragment from pEGFP-C1 (Clontech) into *Sac*I-*Xba*I site of pBI221 vector. GFP

fusion proteins, GFP::M355 and GFP::M404, were constructed by excising cDNA clones covering amino acids 225–309 of M355 and 102–219 of M404, respectively, from pBluescript SK-II plasmid by *Pst*I-blunt/*Kpn*I, and then inserting them into the *Bgl*III-blunt/*Kpn*I site of 221-EGFP-C1 vector. Onion epidermal cells were bombarded with 1.6  $\mu$ m gold particles coated with GFP::M355, GFP::M404 or 221-EGFP-C1 vector using PDS-1000/He particle delivery system (BioRad). Samples were kept at room temperature for 8 hours and the GFP localization was visualized using a fluorescent microscope, E800 (Nikon), equipped with a color CCD camera system (Hamamatsu Photonics).

## Results

### Isolation of cDNAs

Although we have identified 418 cDNA clones, we chose M355 and M404 for further analysis because of the presence of putative NLSs within their predicted amino acid sequences. The full length cDNA sequence was obtained by 5'-RACE using mRNAs obtained from the whole anther at meiotic prophase. The M355 cDNA is 1201 bp in length with an open reading frame encoding 308 amino acids that shows significant homology to a putative protein predicted by the genomic sequences of Arabidopsis and rice (Fig. 1A and Fig. 2). Because of the low abundance of the M355 tran-

A	40	B	40
GGTTAGATTTCCTCTCCGCTCTAACAGCTCACAGC	40	TGCAGTGTGCAAGACTGCTTCAGAGTCCAGACTCGAGTC	40
TGCATCGAGATGGTTCGGAACGGGAGCCGTCGAGGGGATA	80	GCATATCCTCATCCCTCCCGCCCGCGGTGATCGCCGGCAC	80
M V G N G S R R G D T		TCCAATGAAGCGCAAAAATGGACCGGAGTACGAAGAATC	120
CTGGAGTTTCCAGCGGATTAGCAGCCGGAACGTCCTTCGC	120	CACCCATATTACGAATACTCCTCCCTCCTCATCGGGC	160
G V S Q A I S S R N V F A		GCCTCTCCAAGCTCCGCTCCCGTGAGAAAAGTTTCCTCC	200
CGCTCTCGATACCCCTCAAGAAGAAGAGGTTCAGACAAG	160	CCATCGCCGACCGCGTCAACGCCCTCCACCCTCCGCGA	240
A L D T L <u>K K K K</u> R S D K		CCCGACTCCTTCCCTTCTCTGGTCTGGCCGCGACGTC	280
GAGAAGTGC AAGGTCAAGGAGGAACCGGCACCGCAGCTGT	200	TCCGTC AAGATCCAGAACATGCGCCACCGTACCTTGCGG	320
E K C K V K E E P A P H V F		M R H Q Y L G V	
TCTGGGCGCCCGCGCCCTCACCGTCAAATCCGCGCGA	240	TGAAGCAAAAATCCTCCGCCCCTCTTCTCCCTCCGCGC	360
W A P A P L T V K S W A D		K Q K I L R P S S S P S A	
TGTCGAAGACGACGACGACGATGACTACTATGCTACCACC	280	CGCCGCGACTTCGACTGGGACGACGGCGTCAATATCTGG	400
V E D D D D D Y Y A T T		A A D F D W D D G V N I W	
GCCCCGCGTCAGCGTGGGGTGTGCTGAGCAGAAGAAGA	320	CCCACTTCTCCTTACAAGCAGGTCTTCGGCGATCTCG	440
A P P S A W G V P E Q K K S		P N F L L Y K Q V F G D L D	
GCAAGGAGTCCCTGTTCTATAGAGGAGGAAGTCAAG	360	ACCTCGATCCCGATCTCGATTCTGACGATGAGGATGAGA	480
K E V P V A I E E E S A S		L D P D L D S D D E E	
TGAAGATGAAGGTCTTGATGAACCTGATGATGAGGCTGTT	400	ATTTGGGGTGGAAACTGAGGAGGACCCGGATCTGGCGG	520
E D E G L D E P D D E A V		L G V E T E E E T R M L A	
GATGAACAAGCTGACCATCAACTCGAGGTTCTGCCCCAG	440	AGGAGGGTGTGGAGCAGGGGAGGAGCCGCGAGGAGGA	560
D E Q A D H Q L E V P A P A		R R V L E Q G E E A A R <u>R R</u>	
CTGAGCCTGATGTGAAGAACCTCCCGTCCCTTCGGTGCC	480	GGTGAGGGGAGGAGGAGGAGGATTTGGCGGAGGACTG	600
E P D V K K P P V P S V P		<u>V R</u> E E E E E D W A R D W	
CAAAGATACAGAAGGCCAATCTCGAAAAGGAGCTGAAG	520	GCGCCGAGGGAGCGGGAGACGGCGTGGAGGATGAGAGG	640
K D T E R Q L S K K E L K		R R R E R E T A V E D E <u>R</u>	
AAAAAGGAGTTGGCAGAATTAGAAGCTCTTTAACTGAGC	560	AGGGAGAGGTGGAGGAGGGAGGAGGAGGAGGGAGGAGG	680
K K E L A E L E A L L T E L		<u>R E R</u> W R R R E E R R E E E	
TTGGAATGGCAGCTGAAGATAACCAAGCTGCAAAAGCAAAA	600	AGGAGATGGAGTGGAGGAGGAGGATGCTGGTGTATGCAGAT	720
G M A A E D N Q A A K Q N		E M E W R E R M L V M Q M	
TGGACCACAGGTGTTGGCCAGGAGAACTGGAGGAGCAT	640	GGAGCATGAGAAACAGGTGATGCAGATGCAGGCTGAGGTG	760
G T T G V G E E K L E E H		E H E K Q V M Q M Q A E V	
AGTGGAGCGAATGAGAAAAGGAAATGTTCCAGTGGAGA	680	TGTC AAGGTGAGATGCAGATAGCGGGATCTCGCACGGC	800
S G A N E K K E N V P V E S		C Q G Q M Q I A G I L A R L	
GCAAAACATCAAAAAGAGAGCGAAAAGGAGAAACT	720	TGGCTGCCAGTTTTTCGGGGCGGGGCTGGCCGGGGTGA	840
K T S <u>K K K</u> A K K E K L		A C Q F F G A G A G G G D	
GTCCAAGGAGGCTAAAGATTCTAATGAACATCCGAACGGC	760	TGGGGGATGCCGAGCCAGGTTCTGCAAGGTTTGCAGCAG	880
S K E A K D S N E H P N G		G G M P S Q V L Q G L Q Q	
AATGTGCGCATGCAGGTACTAGTGCAGAGGTGACCGCTC	800	GAGCAGGAGCATTACCGGGAAGCTTGGTGGGGATGATG	920
N G A D A G T S A E V T A R		E Q E H S P G S L V G D D G	
GTCCAGTTGATATGAAAGAGCGGCTGAAGAAGATGGCTTC	840	GGAAGATGAGGCTCATCAAGTGAGCACTATCTCTAGAA	960
P V D M K E R L K K M A S		K N G A H S S E H Y L *	
AACGAAGAAGAAATCTAGCAAAGAGATGGACTCTGCTGCT	880	TCATGTAAAGTTGTGTCTCACTAACTTTATCTGTGTTA	1000
T K K K S S K E M D S A A		GAATCTTGTACGGCATAGAAATGTCTGAATGCTTGGATTT	1040
AGGGTGCOCGGTGGAGGCAGCTGCCAGGAGTGCAAGC	920	TC AATGATGAATCAATTTGAACATCTGAAATAAGATAATT	1080
R A A A V E A A A R S A K L		GTTGGTGTATCGGGGATGCAAAATATATAGTTATTTTC	1120
TGGCCGCTGCGAAGAAAAAGAGAAGAGTCATTACAATCA	960	AAAAAAAAAAAAAAAAAAAA	1138
A A A K K K E K S H Y N Q			
ACAGCCAATGCGGTAGGCTGCTGTGCTATCTGTGGCTGG	1000		
Q P M R *			
CTCATGTTGGGACATCGGTGCTAAATAGTGAAGAAAATAT	1040		
C T C T T T T A T T G G T T G A A T G G G A A A T A A A C T G A G T T G T C G	1080		
CACAGATGACCGATGTGTTCTGTGTGGTACTAGTTTTTCC	1120		
T T A G T C T G T C A A T G C C T T G C A T G A A T G T G T A G A A G A T A C	1160		
ATGTGACCATGGTTTCTTAAAAA AAAAAAAAAAAAAAAAAA	1200		
A	1201		

Fig. 1 cDNA and deduced amino acid sequences of M355 (A) and M404 (B). Putative nuclear localization signals are underlined.

scripts, we could not determine the exact size of the mRNA by RNA gel blot analysis. Also, the putative 5'-noncoding region of M355 mRNA did not contain in-frame stop codons, it is possible that the obtained cDNA is not the full-length clone. However, the high similarity to Arabidopsis homolog suggests that the M355 cDNA encodes a full-length protein. The M404 cDNA is 1138 bp in length with an open reading frame encoding 219 amino acids that shows no similarity to previously reported proteins deposited in the database (Fig. 1B). Like M355, we could not confirm the size of M404 mRNA by RNA gel blot analysis, but the presence of stop codons in all three ORFs suggests that the cDNA encodes a full-length protein.

Although neither protein exhibits any significant similarity to previously characterized proteins, searches for functional motifs within the amino acid sequence revealed the presence of clusters of basic amino acid residues that may constitute nuclear localization signals for M355 and M404 (Fig. 1).

*Tissue specificity and temporal accumulation of mRNAs*

Because we could not detect any discrete signals by RNA gel blot analysis, we conducted semi-quantitative RT-PCR analysis for the detection of tissue-specific accumulation of mRNAs for M355 and M404. As shown in Fig. 3, M355 mRNA can be detected in anthers of all stages of microspo-

rogenesis and mature pollen. Although higher expression levels are detectable from the whole anther and PMC samples, the M355 mRNA is also detected from somatic tissues including leaf, stem and root. Therefore, the tissue specificity of M355 mRNA accumulation does not appear to be tightly regulated at the level of transcription. On the other hand, M404 mRNA expression shows a discrete tissue- and stage-specific expression pattern. As shown in Fig. 3, M404 mRNA accumulates at high levels during early stages of microsporogenesis. Although low levels of mRNA accumulation can be detected from somatic tissues, a PMC-specific expression is also evident. These results indicate that the M404 gene expression is associated with male meiosis and is predominantly expressed in PMC.

*Intracellular localization of GFP fusion proteins*

Because the amino acid sequence of both proteins indicated the presence of clusters of basic amino acid residues that may constitute an NLS, we tested the nuclear localizing activity by fusing GFP and expressed in plant cells. Plasmid vectors expressing GFP fusion protein were constructed by fusing GFP at the amino terminus of each protein. Plasmids were introduced into onion epidermal cells by microprojectile bombardment and expressed under control of the CaMV35S promoter. A plasmid construct that expressed GFP alone, designated 221

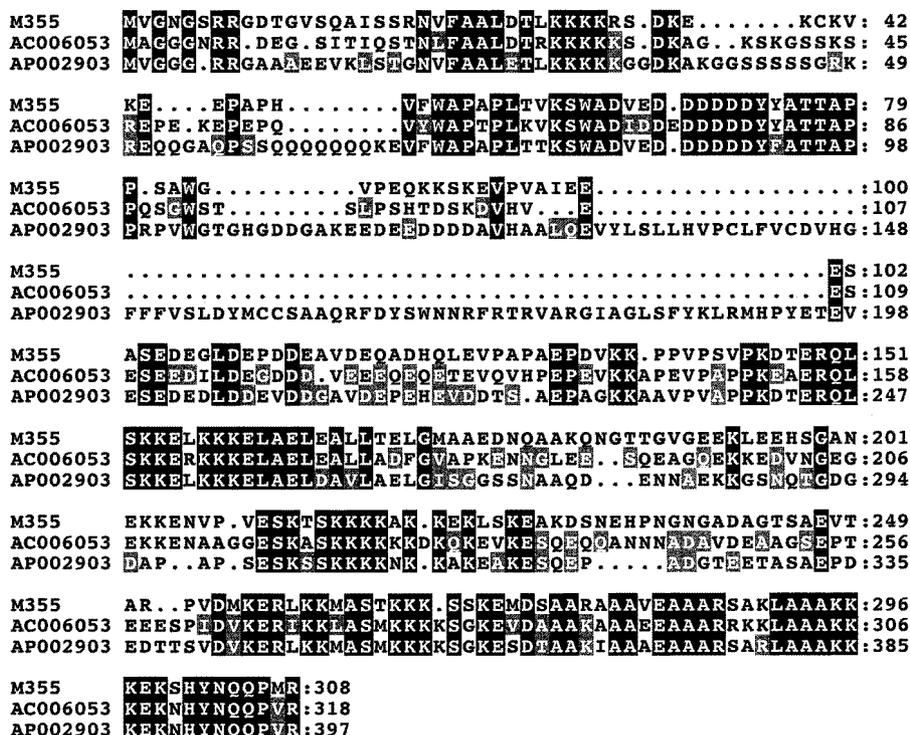
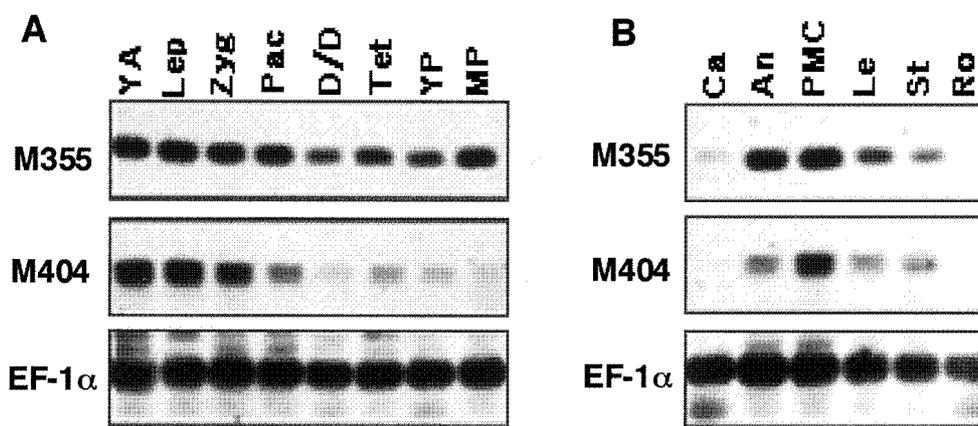


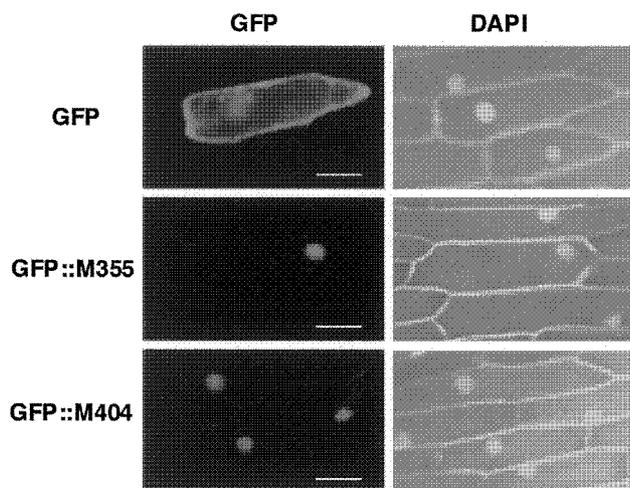
Fig. 2 Sequence comparison of M355 and putative ORFs from Arabidopsis (GeneBank accession number AC006053) and rice (GeneBank accession number AP002903).

Identical and similar residues are boxed in black and gray, respectively. Sequences were aligned using the ClustalW method.



**Fig. 3** mRNA accumulation pattern of *M355* and *M404*.

Reverse transcription was conducted with 0.5  $\mu$ g total RNA from anthers of indicated developmental stages (A) and different tissues (B). PCR was performed with specific primers for *M355* and *M404*. *EF-1 $\alpha$*  was used as an internal control. The PCR products were separated on a 1.2% agarose gel, transferred to nylon membrane, and probed with cDNA fragments corresponding to each of the amplified products. YA, young anther; Lep, leptotene stage; Zyg, zygotene stage; Pac, pachytene stage; D/D, diplotene–diakinesis stages; Tet, tetrads; YP, young pollen; MP, mature pollen; Ca, callus; An, anther; PMC, pollen mother cell; Le, leaf; St, stem and Ro, root.



**Fig. 4** GFP::M355 and GFP::M404 fusion proteins localize to the nucleus.

Onion epidermal cells were bombarded with plasmid vectors expressing GFP, GFP::M355 and GFP::M404, respectively. DAPI images are shown on the left. Intracellular localization of the GFP fusion protein is visualized by fluorescent microscopy 8 hours after bombardment. Scale bars = 100  $\mu$ m.

-EGFP-C1, was used as a control. Fluorescent microscopic observation of GFP signals revealed a clear difference in intracellular localization between fusion proteins and the GFP control. As shown in **Fig. 4**, GFP::M355 and GFP::M404 fusion proteins are targeted to the nucleus, whereas the control GFP protein is localized throughout the cytoplasm. These

results indicate that M355 and M404 proteins are localized to the nucleus when fused to GFP.

## Discussion

Unlike *LIM* genes, *M355* and *M404* were originally identified as cDNA clones from zygotene stage cDNA library without any selection for specific expression. The predicted amino acid sequence of M355 exhibited significant similarities to previously reported putative plant gene products from *Arabidopsis* and rice whose functions are unknown. On the other hand, M404 shares no homology to previously described proteins deposited in databases. However, the presence of basic amino acid clusters found within the amino acid sequence of both proteins suggests that they constitute NLS. The possible translocation to the nucleus directed by the NLS suggests that M355 and M404 might be involved in nuclear events during the progression of microsporogenesis or meiosis.

The results of RT-PCR analysis show that the M355 and M404 mRNAs are preferentially expressed during prophase I of microsporogenesis. M355 mRNA can be detected at high levels throughout the progression of microsporogenesis while M404 mRNA accumulation is more specific to PMCs at early stages. These results suggest that the M404 is involved in early meiotic events during microsporogenesis. On the other hand, because low levels of mRNA accumulation are also observed in vegetative tissues, we cannot exclude the possibility

that both genes are also expressed in vegetative tissue and involved in cellular events other than microsporogenesis or meiosis.

The *LIM* genes have been identified as lily meiosis-specific genes specifically expressed during prophase I of microsporogenesis (Kobayashi *et al.*, 1994). The most critical difference in the gene expression pattern of *M355* and *M404* compared to previously described *LIM* genes is the expression of mRNA in the young anther stage. Because *LIM* cDNAs were selected from zygotene stage cDNA by subtracting cDNAs from young anther, a certain portion of cDNAs for meiosis-associated genes, including *M355* and *M404*, would have been lost during the process of the cDNA selection. Therefore, some of the meiosis-associated genes involved in early stages of meiosis or microsporogenesis would not be identified by the strategy exploited for the isolation of *LIM* genes. We consider it necessary to carry out large-scale sequencing without the subtraction process for the identifying novel meiosis associated genes.

Together with the fact that the GFP fusion proteins localize to the nucleus, data obtained in this study suggest that *M355* and *M404* are nuclear proteins that may play a role in meiotic progression or microsporogenesis. Since the mechanisms of nuclear protein transport during the process of meiosis may be different from those of mitotic cells, it may be necessary to test NLS activity of *M355* and *M404* in a cell undergoing meiosis. However, because of our inability to express high levels of GFP fusion proteins enough to be visualized by fluorescent microscopy, we could not carry out GFP-tag experiments using meiotic cells. On the other hand, the presence of typical NLSs of meiosis-associated proteins suggests that the NLS sequence capable of mediating nuclear transport in mitotic cells is also active in the meiotic nuclear transport system.

Because nuclear events during meiosis are unique, it is possible that specifically expressed genes such as *LIM* genes are involved in nuclear or chromosomal events during meiosis. Previous studies on *LIM* genes suggested that the *LIM15* protein that shows similarity to an *E. coli* DNA recombination protein, *RecA*, is involved in meiosis and plays an important role in meiotic progression (Kobayashi *et al.*, 1993; Terasawa *et al.*, 1995). Also, we have identified the nuclear localizing activity of *LIM5* and *LIM13* gene products both of which harbor NLS and accumulate specifically during microsporogenesis (Ogata *et al.*, 1999). Since *LIM5* and *LIM13* show no homology to previously described amino acid sequences deposited in the database, we

speculate that they might be involved in the nuclear event unique to lily microsporogenesis. Similarly, because no homology was discovered by the database searches of the *M404* amino acid sequence, available evidence suggests the possibility that the *M404* protein is unique to lily and that it is involved in events specific to lily microsporogenesis or meiosis. On the other hand, the presence of putative homologous genes in *Arabidopsis* and rice indicates that the *M355* is a member of a ubiquitous protein that may play a role in microsporogenesis.

Because of lack of the established transformation system and the absence of background for genetic studies in the lily system, the functional studies of *M355* and *M404* proteins are rather limited. However, the availability of the *M355* homologue from *Arabidopsis* and rice would allow further study of biological functions of the protein *in vivo*. On the other hand, detailed immunocytochemical studies will also be necessary for elucidating the cellular events in which *M355* and *M404* are involved. We are currently investigating the intracellular localization of *M355* and *M404* proteins in lily microsporocytes using antibodies raised against recombinant proteins expressed in *E. coli*.

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