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Large-scale Sequencing of Meiosis-associated Genes from a cDNA Library of Lily Microsporocytes

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Abstract

Lily, *Lilium longiflorum* has been used to study meiosis for advantages of the synchronous development among sporogenous cells within an anther. In order to identify genes expressed at meiosis, we carried out random sequencing of a cDNA library which represented the mRNAs from microsporocytes of *L. longiflorum* at the zygotene stage of meiotic prophase. To avoid redundancy of cDNA clones and to obtain novel sequence information efficiently, we exploited a simple and versatile "self-hybridization" strategy. 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.

1. Introduction

Meiosis, one of the most commonly observed and indispensable processes in sexually reproducing eukaryotes, is comprised of a complex series of events that includes chromosome pairing, synaptonemal complex formation and crossing over, chromosome segregation, and cytokinesis. The complexity of events suggests that many genes are tightly regulated to ensure each successful meiotic process. 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 yeast (Hawley and Arbel, 1993; Sthal, 1996). In higher eukaryotes, however, few meiotic genes have been reported because of the lack of appropriate analytical techniques. Lily, *Lilium longiflorum* has been used for decades to study meiosis because of the accessibility of the male gametophyte (microsporocytes and pollen grains), the synchronous development among sporogenous cells within an anther, and the large size of their cells and chromosomes (Erickson, 1948; Hotta *et al.*, 1985). Appels *et al.*, (1982) identified thirteen cDNA clones that were specifically expressed and abundant in meiocytes. The cDNAs contained repeated sequences shared by at least eleven of the cDNAs and were found to be homologous with DNA sequences from wheat, rye, and maize. Kobayashi *et al.* (1994) have isolated cDNA clones which are preferentially expressed during the meiotic prophase of microsporogenesis in *Lilium longiflorum* by subtractive hybridization screening. Isolated cDNA clones were classified into 18 groups and the corresponding genes were named *LIM* (Lily messages Induced at Meiosis). The amino acid sequence for most of the predicted gene products is novel, while some of them show similarity to previously described proteins. LIM15, which is homologous to bacterial RecA protein, is one such example. The LIM15 homologs in yeast and mouse play an important role in meiotic progression (Kobayashi *et al.*, 1993; Pittman *et al.*, 1998; Yoshida *et al.*, 1998). These results indicate that some zygotene-specific transcripts from lily microsporocytes would be important for meiotic processes. However, it has been shown that genes that are expressed in somatic cells 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). Although, the study of meiosis-specific genes provided many important knowledge, these results suggest that it is also important to study genes expressed in somatic cells for the elucidation of the meiotic process.

Random cDNA sequencing can be used to isolate

many functional genes which are 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 (Uchimiya *et al.*, 1992; Sasaki *et al.*, 1994).

Our objective is to obtain information for genes expressed at meiosis in higher plants. Using a cDNA library constructed from mRNA of zygotene stage microsporocytes, we carried out random cDNA sequencing. Here we report a simple and versatile technique for random cDNA sequencing and the provisional results of our sequencing project.

2. Materials and Methods

2.1 Preparation of cDNA fragments from the phage cDNA library

Preparation of plasmids containing cDNA inserts from the phage library prepared from zygotene stage microsporocytes (Kobayashi *et al.*, 1993) was carried out according to the protocol recommended by the supplier (Stratagene). After digestion by *Eco*RI and *Xho*I, the plasmid sample was size fractionated by agarose gel electrophoresis. DNA fragments ranging from 0.4 to 2.0 kbp were excised from the gel and purified by electro-elusion.

2.2 Selection and isolation of lambda phage clone

For the selection of phage clones, the same cDNA library mentioned above was hybridized with radio-labeled cDNA fragments. The bacteriophage library was plated at a density of 2×10^3 pfu per 100 x 140 mm square plate. Plaques were lifted onto Hybond-N+ nylon membrane (Amersham) and prehybridized for 1 hr at 42 ℃ in 50% formamide, 5xSSC (1xSSC is 0.15 M NaCl, 0.015 M sodium citrate), 5% SDS. The probe was prepared by labeling the isolated cDNA inserts with a random primer DNA labeling kit (Takara). The probe was added to the prehybridization mix, and incubated at 42 ℃ for 16 hr. Filters were washed twice for 15 min in 0.1 % SDS, 0.5 x SSC at 65 ℃ and exposed to Fuji XAR film.

2.3 DNA sequencing

Selected phage clones were converted into plasmids and digested with *Eco*RI and *Xho*I to confirm the size of the cDNA fragment. Plasmids with no cDNA insert were excluded. DNA sequencing was carried out using a Perkin Elmer Sequencing kit according to the manufacturer's instructions. Selected cDNA clones were sequenced by cycle sequencing using dye-labeled M13 reverse and -21 M13 primers on an automated DNA sequencer (ABI 373 Stretch, Applied Biosystems Inc.). The database search was done by the net work service of the DNA Data Bank of Japan using the BLAST (blastx) program (Altschul *et al.*, 1990).

3. Results

3.1 Selection of cDNA clones and sequencing

As shown in **Fig. 1**, the entire procedure for "self-hybridization" was simple and required no additional steps other than the conventional plaque hybridization protocol. Hybridization signals resulting from this procedure showed various signal intensities. In this study, however, judging from the signal intensities on X-ray films, 10 % of all phage plaques with a lower signal intensity were designated as weak signal clones while the others were designated strong or middle signal clones. Selected phage clones were converted into plasmids and used as templates for DNA sequencing. Single-run DNA sequence data were obtained from 418 cDNA clones including 115 strong and 303 weak signal cDNA clones. The usable sequence length of each clone was approximately 500 bases.

3.2 Evaluation of the selected clones

In order to confirm the relationship between signal intensity and the ratio of a selected clone in the original cDNA library, we compared the existence ratio among individual clones. We randomly selected six cDNA clones including three of each from a strong signal group and weak signal group. Each cDNA fragment was excised from the plasmid vector, purified and used as radioactive probes for the detection of existence ratio of original phage clones in the cDNA library. Membranes for plaque

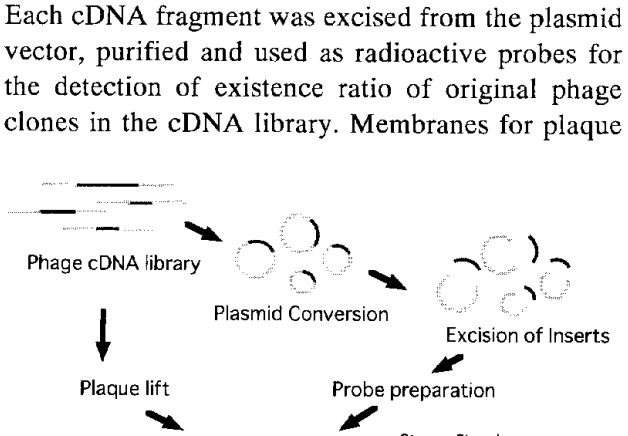

Fig. 1 A schematic representation of the self-hybridization strategy

hybridization were prepared and hybridized with six probes each by the method described above. Then we scored the number of positive signals appeared on X-ray films after autoradiography and the average ratio of each group was calculated. As summarized in **Fig. 2**, a clear difference was observed between weak and strong signals. The result suggests that the signal intensity obtained by this procedure reflected the existence ratio of a cDNA clone in the lambda phage cDNA library used in this study.

3.3 Sequence analysis of cDNA clones

Sequence comparison among cDNA clones obtained in this study revealed relatively higher redundancy among strong signal clones. On the other hand, most of the cDNA sequence derived from a weak signal clone was unique (**Fig. 3**). All sequences were searched for homologies against databases using BLAST (blastx) program (Altschul *et al.*, 1990). Blastx automatically translated all six reading frames of sequences, and compared each translation with the protein sequence database. The deduced amino acid sequence homology between a cDNA clone and a known sequence was deemed significant if the blastx score was greater than 100. Partial nucleotide sequences of 418 cDNA clones were searched for homology. Putative amino acid sequences encoded by 171 cDNA clones, which occupy 41% of the total cDNAs, showed significant

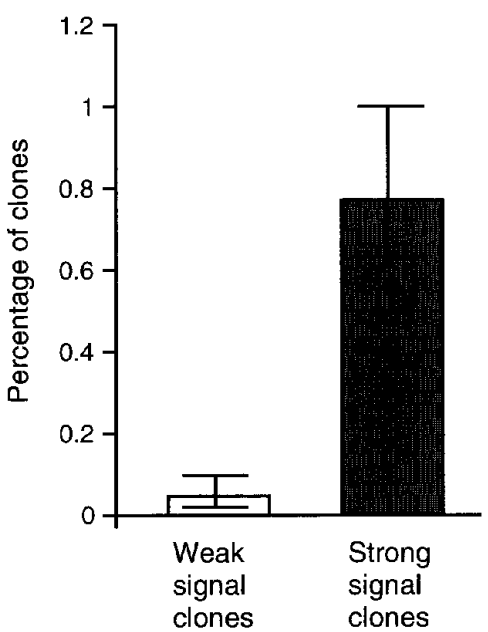

Fig. 2 Comparison of ratio of weak and strong signal clones in the cDNA library
Percentage of each clone was estimated by the number of hybridization signals obtained by plaque hybridization against the original cDNA library.

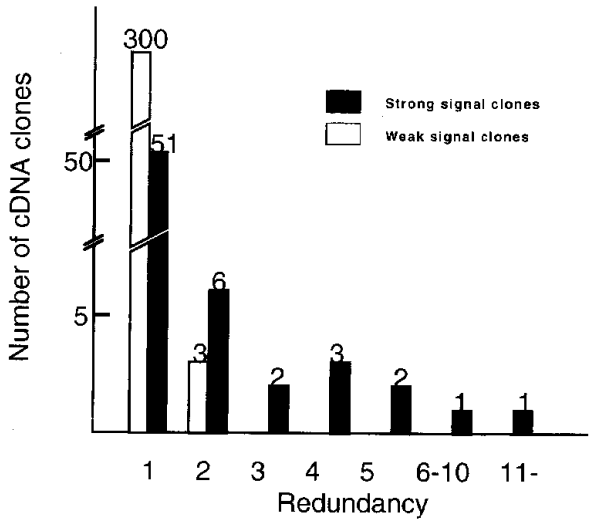

Fig. 3 Redundancy of weak and strong signal clones
Sequence comparison among cDNA clones was carried out by Macintosh DNASIS (Hitachi Software Engineering) program software using 3' non-coding sequence of each clone.

similarity to translation products encoded by nucleotide sequences registered in the databases. For 57% of the strong signal clones, a significant similarity to previously described genes was found. On the other hand, only 36% of the weak signal clones showed a significant database match. The difference between strong and weak signal clones is due to the relative abundance of novel sequences within the weak signal clones. As summarized in **Table 1**, cDNA clones identified in this study are classified into a broad spectrum of house-keeping genes, which function in transcription, translation, glycolytic pathway, tricarboxylic acid (TCA) cycle, nucleic acid synthesis, electron transport system, and photosynthesis. Five cDNA clones encoding gene products homologous with LIM proteins were identified as the weak signal clones.

4. Discussion

Expressed sequence tags (ESTs) have been produced in many species and provided useful and important sequence information of cDNA for molecular biology and plant science. However, it is rather difficult to carry out a large-scale sequencing project in an individual research group.

In order to isolate and analyze cDNA clones efficiently, various methods have been developed. For the identification of specific genes, the subtractive hybridization technique was used efficiently (Kobayashi *et al.*, 1994). Also, an approach has been taken to construct equalized cDNA library for the isolation of cDNA clones of low abundance (Kohchi *et al.*, 1995, Kohchi, 1997). While these

strategies have successfully been used to isolate cDNAs for mRNA of low abundance, the procedure requires experimental skill and is tedious.

On the other hand, the cDNA clone selection method described in this study does not require any special technique. As shown in **Fig. 1**, the procedure does not require any additional steps such as PCR amplification. Moreover, the protocol can be used especially when the mRNA sample is not readily available. Unlike the conventional differential hybridization technique, no mRNA sample is required for the probe preparation in this procedure. Because of the difficulty in harvesting enough amount of tissue which undergoes meiosis, this advantage is particularly important for our research purposes.

As shown in **Fig. 2**, hybridization signal intensity obtained by the protocol used in this study depends on the ratio of each probe prepared from converted plasmid cDNA inserts. Therefore, the signal intensity may reflects the mRNA expression level of a corresponding cDNA clone. However, it also depends on the length and the labeling efficiency of each cDNA probe. For the evaluation of accurate expression levels, further experiments will be required.

In order to carry out random sequencing efficiently and to obtain novel sequence information, it is important to avoid redundancy of cDNA clones to be sequenced. The result shown in **Fig. 3** clearly demonstrates the advantage of selecting weak signal clones to avoid redundant cDNA clones. Conversely, by selecting strong signal clones, it is easy to obtain sequence information of abundant cDNA clones.

Previous studies in the plant random sequencing project revealed that the cDNA clones encoding putative ribosomal proteins were dominant among cDNA libraries and more than 20% of the clones identified in those works were classified into a group of genes functioning in translation processes (Uchimiya *et al.*, 1992, Keith *et al.*, 1993, Höfte *et al.*, 1993, Newman *et al.*, 1994, Sasaki *et al.*, 1994, Hihara *et al.*, 1997). As summarized in **Table 1**, cDNA clones identified in this study exhibited homology to a broad spectrum of genes. The low abundance of cDNAs encoding proteins involved in translation processes is consistent with the fact that transcription and translation activites are drastically reduced in meiocytes (Tabata *et al.*, 1993). Interestingly, LIM cDNA clones obtained in this study were identified as weak signal clones. These results suggest that the cDNA clone selection strategy exploited in this method is useful enough for isolation and identification of novel cDNA sequences in a cDNA library.

Although we have used a cDNA library made from zygotene stage microspores, the use of different cDNA libraries made from various stages of microsporogenesis would enable us to carry out the differential hybridization of the cDNA clones. In addition, the use of a novel technique such as DNA microarray technology that has been developed rapidly, combined with the strategy described in this

Table 1. Functional classification of cDNA clones from zygotene stage microspores of *L. longiflorum*.

	Strong signals		Weak signals	
	No.	(%)	No.	(%)
Database-matched	61		110	
Translation	0	–	7	6.4
Transcription	1	1.6	3	2.7
Glycolytic pathway	8	13	6	5.5
Tricarboxylic acid cycle	0	–	2	1.8
Nucleic acid biosynthesis	1	1.6	2	1.8
Electron transport system	3	4.9	3	2.7
Photosynthesis	0	–	3	2.7
Retrotransposon-related	16	26	23	21
Stress-related	2	3.3	4	3.6
ER-related	4	6.6	2	1.8
Membrane-binding protein	1	1.6	3	2.7
Hypothetical protein	2	3.3	20	18
Meiosis-associated protein (LIM protein)	0	–	5	4.5
Others	23	38	27	25
Not databese-matched	54		193	

study, would allow us to develop a more efficient large scale cDNA sequencing for the identification of functional genes during meiosis I of microsporogenesis.

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