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Genome Analysis of *Lilium tigrinum* by Chromosome Microdissection and Molecular Cytogenetic Techniques

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ABSTRACT

Chromosome microdissection and microcloning are powerful tools for plant genome research. Here we describe the isolation of chromosome #1 derived sequences from *L. tigrinum* with these techniques and their characterization. Detailed chromosome analysis was performed by FISH and then chromosome #1 was isolated from metaphase chromosomes of *L. tigrinum* by microbeam dissection. DOP-PCR and LA-PCR were used to amplify a DNA of chromosome #1 segments. PCR products from the microdissected chromosome were cloned into a plasmid vector to construct a chromosome #1 specific library and sequenced. BLAST-nr revealed that 28% of the sequences were matched with known genes and transposons, and the rest of 72% did not match with known sequences from NCBI database of plant taxa. The unknown sequences were putatively divided into five classes and we called them lily unique unknown repeats. FISH confirmation with some clones confirmed that the products from both methods were indeed amplified from the chromosome #1 of *L. tigrinum* genome. These results provide important information for not only the composition of the *Lilium* genome but also for detailed sequence information of huge genome sized plants.

Keywords: chromosome specific library, DOP-PCR, FISH, LA-PCR, microcloning, universal amplification
Abbreviations: DOP-PCR, Degenerate Oligonucleotide Primed Polymerase Chain Reaction; **FISH**, Fluorescence *in Situ* Hybridization; **LA-PCR**, Linker Adaptor-Mediated Polymerase Chain Reaction; **LUUR**, Lily Unique Unknown Repeats

INTRODUCTION

Chromosome microdissection and microcloning are valuable tools for plant genome research. Initially, chromosomes were dissected with glass micro-needles under an inverted microscope (Scalenghe *et al*. 1981). Afterwards, chromosome microdissection was performed using a computer system controlling a laser microbeam (Monajembashi *et al*. 1986).

Chromosome microdissection and microcloning techniques were first developed by Scalenghe *et al*. (1981). They applied these techniques to separate the polytene chromosome from *Drosophila melanogaster* and obtained 80 clones. At that time, studies were mainly focused on human (Bates *et al*. 1986) or animal chromosomes (Röhme *et al*. 1984; Brockdroff *et al*. 1987). Because plant chromosome preparation is more difficult and needs more elaborate techniques, microdissection was belatedly applied to plant chromosomes. The first use of plant chromosome microdissection was to isolate the B-chromosome from rye (Sandery *et al*. 1991). Until now, microdissection and microcloning have been used to isolate specific chromosomes and chromosome specific fragments in wheat (Albani *et al*. 1993; Liu *et al*. 1999; Hu *et al*. 2004), oat (Chen and Armstrong 1995), maize (Ponelies *et al*. 1997; Cheng and Lin 2003), rye (Zhou *et al*. 1999), citrus (Huang *et al*. 2004), poplar (Zhang *et al*. 2005), soybean (Zhou *et al*. 2001), wild beet (Jung *et al*. 1992), barley (Schondelmaier *et al*. 1993), *Pinus densiflora* (Hizume *et al*. 2001), *Rumex acetosa* (Shibata *et al*. 1999), cotton (Peng *et al*. 2012), and sex-chromosomes in *Silene latifolia* (Scutt *et al*. 1997; Hobza *et al*. 2004). An overview of the studies can be viewed in **Table 1**.

In the beginning, a large amount of dissected chromosomes had to be used for microcloning, because of the low cloning efficiency. However, with the introduction of polymerase chain reaction (PCR) technology, a minimal amount of DNA from a chromosomal region could be amplified by two types of PCRs using different types of primers: (1) degenerate oligonucleotide primed PCR (DOP-PCR) (Telenius *et al*. 1992; Pich *et al*. 1994) and (2) linker adaptor-mediated PCR (LA-PCR) (Albani *et al*. 1993; Chen and Armstrong 1995). The DOP-PCR method, which is rapid, efficient and species-independent, relies on direct amplification along the dissected chromosomes using degenerate primers (Telenius *et al*. 1992). For LA-PCR, chromosomal DNA is digested by restriction enzymes and then ligated to adaptors that provide primer binding sites for PCR amplification (Zhou and Hu 2007).

Detailed genome sequencing analysis studies until recently were applied to angiosperms with small or medium-sized genomes that functioned as model and/or crop plants such as *Arabidopsis* (1C = 125 Mbp) (the Arabidopsis Genome Initiative 2000), rice (1C = 430 Mbp) (International Rice Genome Sequencing Project 2005), *Brassica* (1C = 520 Mbp) (Yang *et al*. 2005), maize (1C = 2500 Mbp) (Schnable *et al*. 2009) and wheat (1C = 5700 Mbp) (Feuillet and Eversole 2007). Recently high-throughput genome sequencing machines are developed, however the giant genome size of several plant species such as *Lilium* (1C = 46,900 Mbp) and *Fritillaria* (1C = *ca*. 127,000 Mbp) are still too difficult for whole genome sequencing research. Therefore it is important to understand the genome composition of such a large-sized plant genome.

In this study, we constructed a chromosome #1 specific DNA library derived from *L. tigrinum* through the microdissection and microcloning techniques. This results presented here will be useful in study of huge sized genome of plant taxa such as the genus *Lilium*.

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Original Research Paper

Table 1 List of previous researches of microdissection and microcloning.

Plant	Target chromosome	PCR	Estimated library size	Reference
Wheat	Chromosome arm	LA-PCR		Albani *et al*. 1993
	Long arm of chromosome 5B	DOP-PCR		Liu *et al*. 1997
	Chromosome 6B	LA-PCR	2.10×10^5~ 2.93×10^5	Hu *et al*. 2004
Oat	Chromosome 21	LA-PCR	5×10^5	Chen and Armstrong 1995
Maize	Chromosome 6	LA-PCR		Ponelies *et al*. 1997
	B-chromosome	DOP-PCR		Cheng and Lin 2003
Silene latifolia	Sex-chromosome	DOP-PCR		Scutt *et al*. 1997
	Sex-chromosome	DOP-PCR		Hobza *et al*. 2004
Rye	Chromosome 1R	LA-PCR	2.2×10^5	Zhou *et al*. 1999
Rumex acetosa	Y-chromosome	DOP-PCR	950 clones (Y-specific)	Shibata *et al*. 1999
Soybean		LA-PCR	2×10^5	Zhou *et al*. 2001
Pinus densiflora	Centromeric segment	DOP-PCR		Hizume *et al*. 2001
Citrus grandis	Chromosome 1	LA-PCR	3×10^4	Huang *et al*. 2004
Popular tremula	Chromosome 1	LA-PCR	3×10^5	Zhang *et al*. 2005
Gossypium arboreum	Chromosome 5	LA-PCR	1.73×10^5	Peng *et al*. 2012

MATERIALS AND METHODS

Plant material, slide preparation and microdissection

A triploid (2n=3x=36) (Noda 1978, 1986; Hwang *et al*. 2011) of *L. tigrinum* Ker-Gawler (syn. *L. lancifolium*) collected from Korean peninsula and maintained *in vitro* was used as the material in this experiment. Root tips of 2~3 cm in length from *in vitro* plant material were collected and pretreated in saturated α-bromonaphthalene (Merck, Darmstadt, Germany) solution at room temperature for 4 h. After pretreatment, root tips were fixed in acetoethanol (acetic acid: ethanol = 1:3, v/v) at room temperature for about 2 h. Immediately after fixation, root tips were treated with an enzyme solution [0.3% pectolyase (Duchefa, Haarlem, The Netherlands), 0.3% cellulase (Duchefa), 0.3% cytohelicase (Sigma, Saint Louis, USA) in 150 mM citrate buffer] at 37°C for 1~2 h. Enzyme treated root tips were squashed on a PALM 1 mm membrane slide. Target chromosomes were separated under a light microscope (Zeiss Axiovert 135, Oberkochen, Germany) using a PALM Robot Microbeam. Separated chromosomes were incubated in a 1 mg/mL Proteinase K solution (solution in nuclease-free water, Promega, Wisconsin, USA) at 37°C overnight. FISH hybridization and detection were followed by Hwang *et al*. (2011).

PCR amplification

1. Optimization of PCR condition

To determine the fine PCR conditions, we tested several different combination of PCR parameters including number of chromosomes (1, 5, 10, 20, 30, 40, 50, and 100), concentration of $MgCl_2$ (1.5, 2.5, and 3.5 mM), *Taq* polymerase (0.5, 0.75, 1.0, and 1.25 units), and treatment time of proteinase K (4 and 24 h).

2. Degenerate oligonucleotide primed-PCR (DOP-PCR) amplification

20 μL of Proteinase K treated chromosome was added in a 30 μL reaction mixture consisting of 5 μL of 10X PCR buffer (TaKaRa, Otsu, Japan), 5 μL of 25 mM $MgCl_2$ (TaKaRa), 4 μL of 10 mM dNTPs (TaKaRa), 4 μL of 10 pM DOP-primer, 0.5 U *Taq* polymerase (TaKaRa), and UV-treated distilled water (The DOP-primer sequence is as follows: 5′-CCGACTCGAGNNNNNNAT GTGG-3′). Amplification was performed in two steps: pre-denaturation at 95°C for 10 min, 5 cycles at 94°C for 1 min, 30°C for 1.5 min and 72°C for 3 min, with a transition time of 3 min between 30°C to 72°C. This step was followed by 35 cycles at 94°C for 1 min, 64°C for 1 min and 72°C for 2 min with an auto-extension step of 14 s/cycle and final extension at 72°C for 7 min.

3. Sau3A1 linker adaptor mediated PCR (LA-PCR)

15 μL of Proteinase K treated chromosome solution, transferred to a 0.2 mL micro-centrifuge tube, was incubated in an enzyme mixture containing 1X enzyme buffer (NEB, Massachusetts, USA) 1X BSA and 1U *Sau3A1* (NEB) at 37°C for 5 h and then inactivation at 80°C for 20 min. *Sau3A1* linker adaptors, with the sequences 5′-GATCCGAAGCTTGGGGTCTCTGGCC3′ and 5′-GGCCAG AGACCCCAAGCTTCG-3′ were ligated to the *Sau3AI*-digested chromosomal DNA. 5 μL of the digested chromosomal DNA was added to a PCR mixture containing 5 μL of 10X PCR buffer (TaKaRa), 5 μL of 25 mM $MgCl_2$ (TaKaRa), 2 μL of 10 mM dNTPs (TaKaRa), 4 μL of 10 pM LA-primer, 2 U *Taq* polymerase (TaKaRa), and UV-treated distilled water up to final volume of 50 μL. Pre-denaturation was carried out at 94°C for 10 min and followed by 35 cycles at 94°C for 45 sec, 67°C for 45 sec, 72°C for 1.5 min and a final extension at 72°C for 5 min.

Library construction

1. Cloning

PCR products from both DOP PCR as well as LA PCR amplified chromosome #1 fragments were purified by use of a PCR purification Kit (Qiagen, Hilden, Germany). 4 μL of purified DNA was ligated into TOPO vector (Invitrogen, California, USA) and incubated at room temperature (22~23°C) for 5~30 min. Ligation mixture was transformed into competent *Escherichia coli* DH5α (RBC, Taipei, Taiwan) by heat shock for 30 sec at 42°C gently. Transformed cell co-cultivated in 250 μL of SOC medium and incubate in the horizontal shaking incubator (200 rpm) at 37°C for 1 h. Cell solution was spread onto pre-warmed LB plates containing 100 μg/mL ampicillin and 50 μg/mL X-gal and incubated overnight at 37°C.

2. Size fractionation by colony PCR

The 15 μL of colony PCR mixture containing small amount of colony, 1.5 μL of 10X buffer, 1.2 μL of 25 mM $MgCl_2$ (TaKaRa), 0.5 μL of 10 mM dNTPs (TaKaRa), 0.5 μL of each M13 forward (5′-GTA AAA CGA CGG CCA G-3′) and reverse primer (5′-CAG GAA ACA GCT ATG AC-3′) (10 pM/μL), 0.1 μL of *Taq* polymerase (TaKaRa), and 10.7 μL of distilled water. Pre-denaturation was carried out at 94°C for 5 min and followed by 35 cycles at 94°C for 30 sec, 58°C for 30 sec and 72°C for 1.5 min and final extension at 72°C for 5 min.

RESULTS AND DISCUSSION

Karyotype of triploid *L. tigrinum* and chromosome microdissection

The most difficult step is dissecting chromosomes because chromosomes are normally tiny and spread on the polyvinyl membrane slide that hinder clear observation under the microscope. Therefore, precise identification of target chromosome is an important step in microdissection. FISH karyotype analysis has to be preceded before isolating target chromosomes. The length of mitotic metaphase chromo-

Table 2 Summary of karyotype and morphological data of triploid *L. tigrinum*.

Chr. number	Chromosome length (μm)			rDNA FISH	
	Short arm (S)	Long arm (L)	Total (S+L)	5S	45S
1	11.98 ± 0.76[a]	16.81 ± 0.23	28.79 ± 0.55	-	S[b]
2	9.37 ± 0.32	15.82 ± 0.45	25.19 ± 0.57	-	S
3	3.89 ± 0.12	15.24 ± 0.30	19.13 ± 0.20	L[c]	-
4	3.39 ± .20	15.63 ± 0.22	19.02 ± 0.39	-	-
5	3.37 ± 0.20	13.96 ± 0.45	17.33 ± 0.57	-	-
6	2.92 ± 0.04	15.21 ± 0.38	18.13 ± 0.37	-	L
7	2.39 ± 0.08	17.26 ± 0.83	19.65 ± 0.75	-	S
8	2.34 ± 0.06	18.83 ± 0.31	21.17 ± 0.25	-	-
9	2.23 ± 0.16	20.44 ± 0.73	22.67 ± 0.86	-	-
10	1.79 ± 0.08	13.78 ± 0.30	15.57 ± 0.30	-	-
11	1.56 ± 0.12	14.67 ± 0.22	16.23 ± 0.34	-	L
12	1.18 ± 0.01	15.99 ± 0.44	17.17 ± 0.44	-	-
Total	46.41	193.64	250.05		

[a]Mean ± standard deviation
[b]short arm
[c]long arm

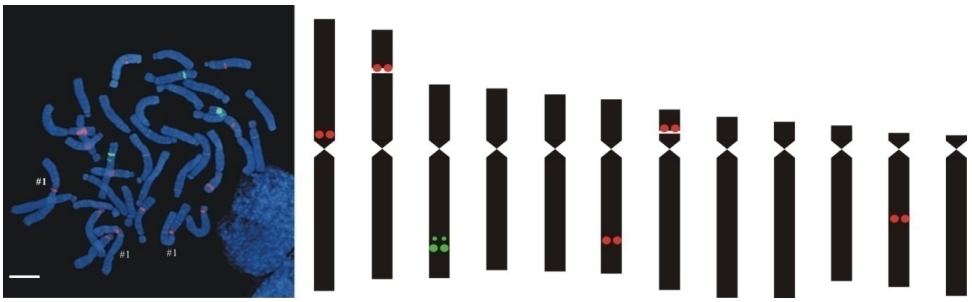

Fig. 1 FISH karyotype analysis of triploid *L. tigrinum*. 5S rDNA and 45S rDNA showed as green and red fluorescence, respectively.

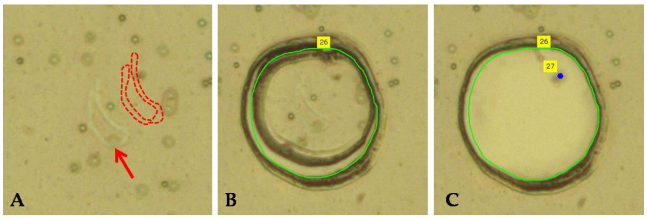

Fig. 2 Serial steps of chromosome microdissection using the PALM system. (**A**) Chromosome observation spread on the polyvinyl membrane slide before microdissection under the PALM system. (**B**) Cutting by microbeam. (**C**) Transferred into micro-tube after cutting.

some ranged from 15.57 (chromosome #10) μm to 28.79 (chromosome #1) μm with a total length of 250.05 μm (**Table 2**). There are fifteen loci of 45S rDNA (chromosome #1, #2, #6, #7 and #11) and two adjacent sites of 5S rDNAs on chromosome #3 in triploid *L. tigrinum* (**Fig. 1**). Chromosome #1 in *L. tigrinum* indicates the largest metacentric chromosome that is relatively easy to discriminate from other chromosomes. In case of triploid, three homologous chromosomes are existed in one cell that is more advantage than diploid cell. After being identified, target chromosome was dissected by microbeam and transferred into 0.5 mL tube containing 40 μL of distilled water (**Fig. 2**). The cutting thickness of razor beam is about 1 μm that means the chromosome size must larger than razor beam. The average size of *Lilium* chromosome #1 is about 25-30 μm that can be cut by razor beam without any trouble. Any of large genome sized plant taxa would be the same condition as done by *Lilium* chromosomes for microdissection. Recently high-throughput genome sequencing machines are developed and numerous sequencing projects are running but, not yet such a huge-genome size plants such as *Lilium* and *Fritillaria*. There is bottle-neck for the large-sized genome of plant taxa in genome sequencing due to cost and time demanding.

PCR conditions: optimization

In the case of DOP-PCR, PCR condition optimization must be preceded before amplification. On the contrary, conditions of LA-PCR were less sensitive than DOP-PCR. Especially, the concentration of $MgCl_2$ and *Taq* polymerase is known to be a key factor for amplification (Telenius *et al*. 1992; Hwang *et al*. 2011). The optimal concentration of $MgCl_2$ and *Taq* polymerase was 2.5 mM and 0.5 U, respectively, with sufficient DNA amplification and no amplification of negative bands (**Table 3**) (Hwang and Lim 2011). Although all tested chromosome copies (1, 5, 10, 20, 30, 40, 50, and 100) were amplified, to avoid a contamination, 10 copies of chromosome may get good results. More than 10 copies of chromosome are exposed to the contamination during microdissection (Hwang and Lim 2011). For the amplification of chromosomal DNA as a template, it needs a thoroughly removal of histone protein from microdissected chromosome. The optimal treatment time of proteinase K was 24 hours, whereas no amplification for 4 hours (Hwang and Lim 2011). Contamination is another bottle-neck which mainly from extraneous DNA during PCR amplification because in the DOP- and LA-PCR, there are no limitations to target regions. To eliminate extraneous DNA, all reagents and equipment are autoclaved and UV-treated if possible. In addition, all materials should avoid long term storage and negative checks during PCR procedure should be carefully screened for bands.

Sequence analysis

We obtained 2,038 clones from two types of PCRs. The insert size of the clone obtained from DOP- and LA-PCR was distributed from 100 to 1700 bp and from 100 to 900 bp, respectively (Hwang and Lim, 2011). A total of 2,038 clones out of 2,112 clones were sequenced. The total length was 3.0 Mbp and an average read length was 742 bp (GenBank ID. HN269094.1~HN270091.1). BLAST-N analysis revealed that 28% of total sequences were known

Table 3 Optimization of DOP-PCR condition for microdissected chromosome #1 of *L. tigrinum*.

PCR parameters	Amplification conditions	Optimized condition for the best results	Results
Magnesium chloride (mM)	1.5, 2.5, 3.5	2.5	2.5 mM $MgCl_2$ gave rise to the best amplification
Taq polymerase (units)	0.5, 0.75, 1.0, 1.25	0.5	High concentration above 0.5 U resulted in increased unwanted negative amplification
Number of chromosomes	1, 5, 10, 20, 30, 40, 50, 100	10	More than 10 copies of chromosome are exposed to the contamination during microdissection procedure
Treatment time of proteinase K (h)	4, 24	24 h	Chromosomal DNA successfully amplified after 24 h treatment of proteinase K

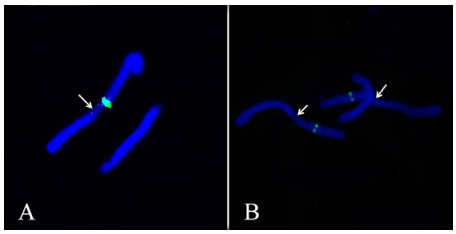

Fig. 3 FISH with DOP-PCR products on metaphase chromosome of *L. tigrinum*. White arrows indicate centromere position. Two randomly selected clones (green fluorescence) from chromosome #1 specific library were detected on long arm (**A**) and short arm (**B**) of chromosome #1.

Table 4 Lily unique unknown repeat sequences divided into five types.

Classification	No. of hits (%)	
Type I (LUUR*1)	1,334	(57)
Type II (LUUR2)	140	(6)
Type III (LUUR3)	201	(9)
Type IV (LUUR4)	172	(7)
Type V (LUUR5)	108	(5)
Total	2031/2330	(84/100)

*LUUR: Lily unique unknown repeat

sequences as genes (21%) or transposon (7%) but, 72% are not matched with known sequences from public domain. At least 57% of total sequences are assumed to be repeat sequences. Twenty-two percent of unknown sequences are not classified indicating that large portion of sequences is unique in *Lilium*. Based on sequence similarity, almost 70% out of unknown sequences are redundant repeats that classified into 5 different classes, and called lily unique unknown repeats (LUUR) (**Table 4**). These repeats are composed of about 80 bp tandem repeats and have *ca*. 90% sequence similarity. The class I repeats represented approximately 65.7% (1,334 sequences) of the total number of unknown repeat elements (2,031) indicating that this element is a major repeat in the chromosome #1 of the lily. The data may imply that a large volume of lily sequences is unique among the huge number of lily genome sequences.

Sequencing results from microdissection are a powerful for linking BAC clones to specific chromosomes and for marker development. Although gene mapping studies are actively being carried out, it is difficult to cover the whole genome alone. Chromosome-specific libraries may apply to the conjunction of the genetic linkage maps and physical maps, offering an understanding of chromosome specific regions of the genome. The result is also valuable in screening various cDNA libraries for isolating expressed sequences from the region.

Chromosome confirmation by FISH

Plasmid DNA from 100 clones selected randomly was labeled with DIG and used for FISH experiments. Two clones displayed clear signals at the long or short arm region in chromosome #1. One displayed strong signals at the long arm of the chromosome #1 (**Fig. 3A**). The other hybridized on the short arm of chromosome #1 (**Fig. 3B**). Two signals are clear and uniquely detected on chromosome #1, therefore it is proved that microdissection, PCR and following steps were properly carried out originated from chromosome #1. As well as, DNA clones isolated from microdissected chromosomes were sufficient to use chromosome specific marker.

Chromosome microdissection and microcloning techniques are efficient tools that incorporate with cytogenetics and genomics biology. These combined techniques were adapted for human genetics in early stage, but later applied for plant genomics. The most important steps for chromosome specific library construction are specific chromosome dissection, amplification of anonymous regions, cloning of fragments and sequencing of clones. Also avoidance of contaminations is of the outmost importance. Some future prospects for micro-dissection in lily and comparisons to the use of the technique in other crops would make the review paper more interesting for a general audience.

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