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Full Length Article

Random PCR of Micro-dissected Chromosome Amplified Predominantly Repeated DNA in *Lilium tigrinum*

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Abstract

Large genome size in plants is largely caused by presence of repetitive DNA, which makes it of great interest to study the chromosome specific markers and function of complex large plant genome. In order to investigate evolutionary patterns of repetitive DNA, random PCR was carried out for a comprehensive repeat characterization and amplification in micro-dissected and micro-cloned *L. tigrinum.* The isolated chromosome was amplified by using DOP-PCR (degenerate oligonucleotide primed PCR) and LA-PCR (linker adaptor-mediated PCR) by which 100 to 2500 bp smear fragments of DNA, and 300 to 2000 bp predominant fragments were obtained. To construct DNA library of chromosome 1, obtained PCR products were cloned in the plasmid vector. The size of cloned inserts varied in DOP-PCR from 100-1700 bp and100-900 bp in LA-PCR, with predominant fragment at 500 to 1000 bp. Based on the BLAST-X search, 28% were matched with protein coding genes in the public database, while 72% of the sequences didn't exhibit any significant similarity with sequences already present in the plant database making up to 72% of unique sequences in the huge lily genome. Two clones obtained from micro-dissected chromosome were successfully detected on the chromosome 1 of diploid and triploid *L. tigrinum*. Among 513 genomic DNA sequences searches, 405 SSRs with perfect repeats were clearly verified and categorized based on number of repeats and their motifs. Six types of repeats were identified, among them penta-nucleotide repeats occurred at the high frequency.

Keywords: DNA library; FISH; LA-PCR; Micro-beam; Micro-cloning; Micro-dissection; SSR markers
Abbreviations: DAPI: 4′,6-diamidino-2-phenylindole; 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 repeat; SSR: Single sequence repeat

Introduction

Genus *Lilium* is one of the interesting materials for genome research because of their huge genome size, its haploid DNA contents (1C) ranges from 13.70 pg in *L. amabile* to 47.90 pg in *L. canadense* (Zonneveld *et al*., 2005; Anderson *et al*., 2010). Variation in genome size is mostly due to divergence in the amount of repeated DNA sequences e.g. the calculated genome size of genus *Lilium* is about 46,900 Mbp, which is approximately 375-times larger than *Arabidopsis* (1C=125 Mbp). Because little information is known about repetitive sequences and molecular markers in genus *Lilium*, it has been difficult to analyze the genome research. *L. tigrinum*, belongs to the *Sinomartagon* section that exists wildly as diploid (2x=24) or triploid (3x=36), making it is excellent tool for chromosome research (Hwang *et al.,* 2011).

Development of flow sorting and chromosome micro-dissection technique can lead to successfully isolate plant chromosomes. Apparently flow sorting technique was used to isolate well-defined large chromosomes such as wheat (Kubaláková *et al.,* 2002) and maize (Li *et al.,* 2004). Unlike flow sorting, micro-dissection technique can be used to isolate single chromosome, small sized chromosomes, and chromosome segments. Although limited chromosomes are isolated by micro-dissection technique, PCR technology can be applied to amplify the large amount of DNA from the randomized region of the dissected chromosome. With the development of micro-dissection and micro-cloning techniques (Scalenghe *et al.,* 1981; Zhou and Hu, 2007), they were used as an effective method aiming for the development of DNA libraries (chromosome-specific) in human (Hadano *et al.,* 1991; Guan *et al.,* 1992) and animals (Greenfield, 1987; Zimmer *et al.,* 1997). Since difficulty of the fine preparation in plant chromosome, this technique

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has so far delayed to apply for plants compared with human and animals. The first application of micro-dissection in the plant was to isolate B-chromosome from rye (Sandery *et al.,* 1991). In the beginning, it was adopted only in limited chromosomes such as satellite- or B-chromosome (Zhou *et al.,* 1999; Cheng and Lin, 2003). Library construction using micro-dissected chromosome has been completed in several plants, such as wheat (Liu *et al.,* 1999), soybean (Zhou *et al.,* 2001), rye (Zhou *et al.,* 1999), maize (Stein *et al.,* 1998), oat (Chen and Armstrong, 1995), barley (Schondelmaier *et al.,* 1993), beet (Jung *et al.,* 1992), Lily (Hwang and Lim, 2011) and poplar (Zhang *et al.,* 2005).

Constructing chromosome-specific library is an efficient approach for understanding specific individual chromosome and the comparative genome analysis of target species. This technique can also be applied for the development of markers specifically for chromosomes and can play a key role in integrating a genetic linkage and physical maps. In addition, micro-dissected DNA clones can be used as chromosome painting probes; it is an important tool for studying of chromosome organization and identification (Zhou and Hu, 2007).

Two methods, DOP-PCR and LA-PCR, have been used to amplify such a small amount of DNA from micro-dissected chromosomes. In DOP-PCR method, degenerate oligonucleotide primer excites priming at multiple loci along the template, which is rapid, efficient and species-independence (Telenius *et al.,* 1992). As an alternative method, LA-PCR, micro-dissected chromosomal DNA is digested by restriction enzyme and then ligating them to specific linker sequences that provide a linker primer binding site to amplify a genome wide randomized sequence.

Simple sequence repeats (SSRs) or microsatellites of short DNA sequences are reported scattered in abundance among the eukaryote's genomes. These sequences of repeated DNA occurred by the mutational effect of replication slippage and amplification of this genomic region by PCR shows polymorphism due to differences present in the repeat units (Tautz and Schotterer, 1994). A DNA marker system based on SSRs proved efficient due to co-dominant, highly variable and allele-specific nature of the markers (Gupta *et al.,* 1996).

In this study, we successfully established a chromosome micro-dissection, micro-cloning and construction of chromosome 1 specific library in *L. tigrinum*. Fluorescence *in situ* hybridization (FISH) technique was adapted to ensure the localization of DNA fragments, which also confirmed as chromosome specific markers. Recombinant clones were sequenced, which could provide to get useful information about studying of *L. tigrinum* chromosome 1.

Materials and Methods

Plant Material, Chromosome Preparation and Micro-dissection

Triploid (2n=3x=36) of *L. tigrinum* collected from Gyeongsangbuk-do, Korea and then grown under *in vitro* conditions was used for present experiment. Fresh root tips were harvested and treated with saturated solution of α-bromonaphthalene at room temperature for three hours and then fixed in 1:3 (v/v) solutions of acetic acid and ethanol. Mitotic chromosome preparation was executed as adopted by (Lim *et al.,* 2001). Chromosome micro-dissection and collection were carried out on microscope (Zeiss Axio-observer Z1) at 400X magnification that was equipped with a micro-beam (PALM). The chromosomes on a slide were lifted up by laser beam and collected in an eppendorf tube (0.5 mL) having 40 µL ddH_2O. Isolated chromosomes were incubated at 37°C for overnight in Proteinase K solution and then inactivated at 80°C for 20 min.

PCR Amplification

DOP-PCR amplification was done in eppendorf tube with 20 µLofchromosome solution, 5 µL of 10x buffer (Takara Shuzou, Siga, Japan), 5 µL of 25 mM $MgCl_2$ (Takara Shuzou, Siga, Japan), 4 µL of 10 mM dNTPs, 4 µL of 10 pM degenerate primer [5'-CCGACTCGAGNNNNNNATGTGG-3'(6-MW)] (Telenius *et al.,* 1992), 0.5 U *ExTaq* polymerase (Takara Shuzou, Siga, Japan) and UV-treated ddH_2O to make final volume 50 µL. After this denaturation of samples was carried out by following the protocols of Hwang and Lim (2011). For LA-PCR, *Sau*3A1 specific linker with the sequences5'-GATCCGAAGCTTGGGGTCTCTGGCC-3' and 5'-GGCCAGAGACCCCAAGCTTCG-3' were used for construction of the *Sau3*A1 linker adaptor (Lowe *et al.,* 2007). The PCR amplification was used in the tube containing 5 µL of linker-ligated DNA, 5 µL of 25 mM $MgCl_2$, 5 µL of 10x buffers, 2 µL of 10 mM dNTPs, 2 U *Ex Taq* polymerase, 4 µL of 10 pM primer and ddH_2O to 50 µL. After denaturing at 94°C for 10 min, PCR was carried out with 35 cycles for 45 sec. at 94°C, 45 sec. at 67°C, 1.5 min at 74°C, followed by final extension for 5 min at 74°C. To examine the DNA contamination, negative control without DNA was used every step of amplification procedure. As well as, genomic DNA from *L. tigrinum* was used as positive control.

Development and Characterization of Chromosome 1-Specific DNA Library

PCR products were refined through DNA purification kit (Qiagen, Germany) followed by ligation into the T vector (Invitrogen, USA). Ligation mixture was transformed into competent cells (*Escherichia coli*, DH5α) by heat shock. Transformed cell co-cultivated in SOC-medium (2% bactotryptone, 10 mM NaCl, 0.5% yeast extract, 10 mM $MgCl_2$, 2.5 mM KCl, 20 mM glucose, 10 mM $MgSO_4$,) at 37℃ for 60 min. Cell solution was plated onto ampicillin (100 $mg \cdot L^{-1}$)–IPTG–x–gal plate for a selection of white colony and incubated overnight at 37°C. To select the recombinant clone size larger than 300 bp, obtained clones after DOP and LA-PCR were PCR amplified using M13- or

linker-primers, respectively. The insert size was measured in agarose gel (1.2%) using DNA ladder USA] and finally 2112 obtained clones were sequenced.

SSR Marker

SSR-containing clone sequences were searched by using software, WebSat (Martins *et al.,* 2009). A recursive criteria for minimum repeat number and motif length was set for 5 monomeric, 5 dimeric, 3 trimeric, 3 tetrameric, 2 pentameric and 2 hexameric nucleotide repeat.

Fluorescence *in situ* Hybridization (FISH)

Plasmid DNA was separated through Qiagen plasmid kit (Germany) and digested using *Eco* RI. After electrophoresis, insert DNA was extracted from the gel and then purified by a Qiagen gel extraction kit (Germany) for probes labeled with digoxygenin-11-dUTP (Roche, Germany). After treatment of RNase (100 $\mu g \cdot mL^{-1}$) at 37°Cfor 30 min and then slides were post-fixed in para-formaldehyde solution (4%) for 10 min. The probe mixture contained 10% dextransulphate, 50% deionized formamide, 2x SSC and 20 $\mu g \cdot mL^{-1}$ of probe DNA before denaturation mixture solution at 70°C for 10 min and cooled on ice. 40 µL of probe mixture was put into the glass slides and then covered with cover slip. The slides were kept for 5 min at 80°C and incubated at 37°C overnight in a humid chamber. The slides were washed for 40 min in 0.1x SSC at 40°C after hybridization, then biotin and digoxygenin were detected through FITC conjugated anti-digoxygenin antibodies (Roche, Germany) and streptavidin Cy3 (USA), respectively. The slides were then counterstained with 2 $\mu L \cdot mL^{-1}$ of 4', 6-diamidino-2-phenylindole (DAPI) in vecta shield (Vecta laboratories Inc., USA) and observed under the Olympus BX61 fluorescence microscope. Images were analyzed using software (Applied Imaging Corporation, Genus version 3.8 program, UK).

Results

Chromosome 1, the largest metacentric chromosome, was easily discriminated at mitotic metaphase cells without any staining and any molecular cytogenetic techniques employed. PCR amplification of the micro-dissected chromosomes yielded products ranged from 100-2000 bp in DOP-PCR and 100-2500 bp in LA-PCR, respectively with predominant fragments size from 300 to 2000 bp, while positive control DNA was amplified between 100 to 3000 bp in both PCR methods. Negative control was not detected in the PCR process; it means that chromosome 1 successfully amplified without any exogenous DNA.

Development and Characterization of Chromosome 1 DNA Using PCR Products

Products of DOP- and LA-PCR from amplification of isolated chromosome1 were used for transformation. DNAs between 300 to 2,000 bp were eluted from the gel and cloned. DOP-PCR gave larger sized recombinant clones than LA-PCR, where DOP-PCR produced 100 to 1700 bp DNA fragments, whereas, it was 100 to 900 bp with LA-PCR.

A total of 2038 clones out of 2112 clones were sequenced with a total length of 3.0 Mbp and an average read length of 742 bp (Table 1). Based on the BLAST-X search, 28% were matched with protein coding genes in public database and 7% out of the 28% corresponded with transposable elements (Table 2). Only 21% showed significant hits with functional genes, among them the hypothetical gene of *Vitis vinifera* showed best hits (Table 3). Among the 7%, which showed significant similarity with the known repeats, *Del-1* retro transposon was most redundant (Table 4). Overall, 72% of the sequences didn't reveal any significant similarity with already known sequences in a public database that reflected that up to 72% of lily sequences are unique in the huge lily genome sequences. By classification of the lily-unique sequences, we could identify highly redundant lily-unique repeat classes which cover 50% of the sequences (Table 4). These showed no significant sequence similarity with known repeats and thus we called lily unique unknown repeats (LUUR). These were classified into 5 different classes and the sequence in each class has somehow shown sequence similarity. Further research should be conducted to characterize the complete feature of these repeat elements. However, the class I repeats represented approximately 30% of the obtained sequences (1,334 sequences out of 4,034) indicating these elements are major repeats in the chromosome 1 of the lily.

SSR Marker Analysis

From 513 genomic DNA sequences searched for the SSRs, 1263 sequences were identified to contain SSRs. Among these sequences, 109 carried more than two SSRs. In total, 405 SSRs with perfect repeats were scrutinized and categorized on the basis of their motifs and number of repeats (Table 5). Among six identified repeats types, penta-nucleotide repeats occurred at the highest frequency (63.7%), although most of them had the repeat number of 2. Only three sequences contained monomeric repeats with repeat number of higher than 10. The motif arrangement of SSRs was observed as highly diversified in nucleotide sequence and repeat number therefore difficult to classify them.

FISH of Recombinant Clones

Clone #40 selected from type I detected a clear signal at the short as well as long arm regions in chromosome 1 of both triploid and diploid *L. tigrinum* Fig. 1a and 1b). Clone #365 (red fluorescence, red arrow) was clearly detected in the interstitial part of short arm of the chromosome 1. Similarly, clone #40 (green fluorescence, white arrow) was observed at long arm of chromosome 1, which was located on

Table 1: Total sequences obtained from the library

Sequence	No. of reads	Total length (bp)	Average of read length (bp)
Raw sequences	4,224	5,637,881	1,335
Trimmed sequences	4,076	3,026,112	742

Note; Trimming: remove vector sequences and raw quality sequence by trim cutoff 0.06

Table 2: Trimmed nucleotide sequence based on BLAST-X

Remarks		No. of reads (%)	
Match	Transposon	299	(7)
	Genes	859	(21)
No match	Unique repeats	2,031	(50)
	No classification	887	(22)
Total		4076	(100)

Note: Match: E value < 0.01

Table 3: Number of plant species showing best match to protein

Species	No. of hits (%)	
Arabidopsis	24	(3)
Oryza sativa	47	(5)
Vitis vinifera	224	(26)
Lotus japonicus	9	(1)
Others	555	(65)
Total	859	(100)

Oryza sativa include relative species of *Sorghum bicolor* (7) and *Zea mays* (4)

Table 4: Classification of repeat elements

Classification		No. of hits (%)	
Retrotransposons		268	(12)
	LINE elements	3	(0)
	Del-1	40	(2)
	Others	225	(10)
Transposable elements		31	(1)
	TNP2	10	(0)
	Transposase	21	(1)
Lily unique unknown repeats		2,031	(87)
Total		2,330	(100)

centromere. Two signals were clear and uniquely detected on chromosome 1; therefore these results showed that the two clones originated from chromosome 1 of *L. tigrinum* through micro-dissection. The same probes were applied to *L. formosanum*, *L. colosseum*, and an Oriental Hybrid which belongs to the sections *Leucolirion*, *Sinomartagon* and *Archelirion*, respectively. Only clone-40 was detected at long arm of chromosome 1 of *L. formosanum* (Fig. 1c).

Discussion

The micro-dissection and micro-cloning has lagged behind in the study of plants, because chromosome preparation is more difficult in plants than humans or animals (Zhou and Hu, 2007). In the beginning, it was adopted only in limited chromosomes such as satellite- or B-chromosome (Zhou *et al.,* 1999; Cheng and Lin, 2003). Well spread chromosome preparation technique without any overlapping is one of the important factors in micro-dissection methods as well as accurate separation of the target chromosome also plays a significant factor. We already conducted accurate karyotype analysis in *L. tigrinum* in order to identify of chromosome 1 before micro-dissection.

Large genome size in plants is largely caused by presence of repetitive DNA, which makes it of great interest to study the chromosome specific markers and function of complex large plant genome. Quality of genomic libraries can be determined by recombinant clones and as well as the size of the DNA fragments inserted (Zimmer *et al.,* 1997), therefore we thought that size-fractionation would give more a higher library. Initially we obtained approximately 3,500 recombinant clones. After size-fractionation, removed the insert size under 300 bp, finally library consists of 2112 clones. In the first attempt to micro-cloning, a large number of isolated chromosomes were directly cloned in rye (Sandery *et al.,* 1991). The clone efficiency has been improved with the application of PCR techniques. Previous researchers reported that the efficiency of cloning for PCR was ranged from 2×10^4 to 5×10^5 (Jung *et al.,* 1992; Chen and Armstrong, 1995). Contrarily, ~3.5×10^3 recombinant clones were produced in present study assuming that there are about 2.2×10^4 recombinant clones harvested without any redundancy. So far, for the construction of the library, most of the researchers conducted two round PCR, whereas in our first attempt, two round PCR reaction caused an increase contamination rate and clone redundancy.

Application of microsatellite markers in taxonomy and genetics studies have been reported by many breeders (Jewell *et al.,* 2006; Gong *et al.,* 2008). Identification of SSRs from DNA sequences of *Lilium* can facilitate the development of PCR-based microsatellite markers and in developing genetic linkage map of important traits in this species (Yamagishi 1995; Abe *et al.,* 2002). Unlike expressed sequence tag (EST)-derived SSRs in which the most abundantly discovered motif is a tri-nucleotide repeat (Morgante *et al.,* 2002; Subramanian *et al.,* 2003; Park *et al.,* 2005), data mining of genomic DNA sequences of *Lilium* in this study revealed that the majority of SSRs had relatively long motif length of penta- (63.4%) or hexa-nucleotide (22.7%) at the given recursive criteria. In the previous report for cotton (Park *et al.,* 2005), the level of allelic variation at SSR loci increased as motif length and the number of repeat unit increased. Currently, interactions between motif lengths, repeat's number and polymorphism levels among different lily cultivars or species are being evaluated by using PCR primer sets franking the SSRs.

Two clones obtained from micro-dissected chromosome 1 were successfully detected on the mitotic metaphase chromosome 1 of diploid and triploid *L. tigrinum*. This result convinced that these clones were originated from chromosome 1. Two clones were also applied to the other species and showed on the chromosome

Table 5: Distribution of the types of repeat among 405 SSRs identified from 513 clone sequences

Motif length	No. of repeats									
	2	3	4	5	6	7	8	9	>10	Total
Monomeric									3	3
Dimeric				12	7	2		3		24
Trimeric			6	1	1					8
Tetrameric	1	16	2							19
Pentameric	257		1							258
Hexameric	92	1								93
Total	350	17	9	13	8	2	0	3	3	405

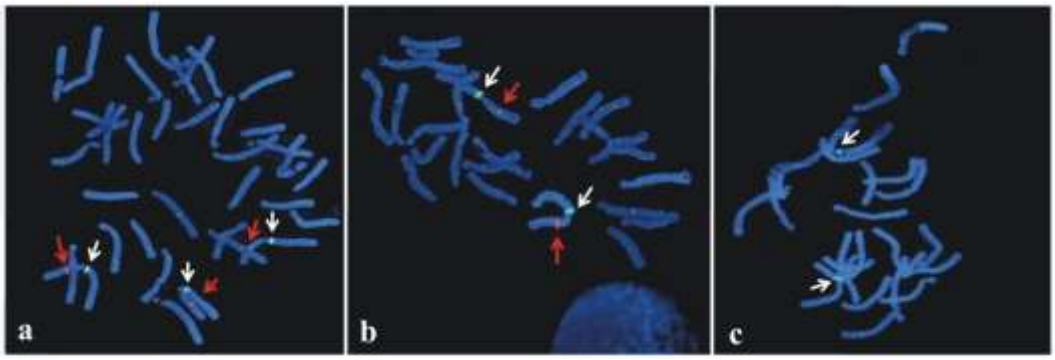

Fig. 1: Fluorescence *in situ* hybridization (FISH) of the DOP-PCR products to triploid- (a) and diploid (b) *L. tigrinum*, and *L. formosanum* (c) somatic metaphase chromosome. Clone # 365 (red fluorescence, red arrows) and Clone #40 (green fluorescence, white arrows) signals distinctively detected on short arm and long arm of chromosome #1, respectively. Whereas in the *L. formosanum* only clone #40 was detected close to the centromere (green fluorescence, white arrows)

1 in *L. formosanum* belonging to section *Leucolirion* but, not in *L. callosum* belonging to section *Sinomartagon*. Overall in present results, DNA clones isolated from micro-dissected chromosomes 1 were indeed confirmed as chromosome specific markers, as well as it is possible to understand phylogenetic relationship between other lily species.

Based on the sequencing result of micro-dissected chromosome, it is concluded that chromosome 1 of *L. tigrinum* consisted of large portion of repetitive sequences. These repetitive sequences were unique in *Lilium* species and were divided into 5 different types. The majority of unknown repeats were LUUR-type I, comprising 57% of the total unknown repeats, in which clone # 40 and # 365 are detected on chromosome 1 not only in *L. tigrinum* but also in other lily species. Clone #40 which belongs to LUUR-type I was detected at the same position of chromosome 1 in *L. tigrinum* and *L. formosanum* simultaneously, although *L. formosanum* belongs to different section.

Conclusion

In the present study, we have successfully established the single chromosome micro-dissection and micro-cloning in *L. tigrinum*. The micro-dissected clones were confirmed by FISH analysis as well as characterization of repeats from sequenced data. The chromosome library of repetitive DNAs is a novel approach for whole genome sequencing programs and presented results could be applied to other eukaryotic genomes. Also, the methods could be valuable for not only constructing physical and genetic maps but also for revealing of useful gene sequences directly from known chromosomes.

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