Chromosome Microdissection, Cloning and Painting of the Chromosome 1 in Poplar (*Populus tremula*)

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(Received 13th May 2005)

Abstract

The chromosome microdissection, cloning and painting technology has evolved into an efficient tool for genomic research. Application of these techniques has rarely been applied for forest plants, largely due to the difficulty of chromosome preparation. The present study was performed to establish a method for single chromosome microdissection, cloning and painting in forest plants using poplar (*Populus tremula*) as a model. An individual chromosome 1 was microdissected from the metaphase spreads of poplar root-tip cells with fine glass needle controlled by a micromanipulator. The dissected chromosome was amplified *in vitro* by the *Sau*3A linker adaptor mediated PCR (LA-PCR) technique, by which 200bp to 3,000bp smear DNA fragments were obtained. Then, the second round PCR products from the single chromosome 1 were cloned into T-easy vectors to generate a DNA library of the chromosome 1. Approximately 3 x 10^5 recombinant clones were obtained. The second round PCR products were used as a complex probe mixture for fluorescent *in situ* hybridization (FISH) on the metaphase spreads of poplar. Hybridization signals were observed, mainly, along the entire chromosome 1, at the same time, signals were also present on telomeric and centromeric regions of other chromosomes. Therefore, this research suggests that chromosome microdissection, cloning and painting of the single small chromosome in forest plants are feasible.

Key words: Chromosome painting, Fluorescent *in situ* hybridization (FISH), Microdissection, *Populus tremula.*

Introduction

Forest trees are the dominant life form in many ecosystems. They provide structural and functional habitat for two-thirds of the Earth's terrestrial species and contain greater than 90% of all terrestrial biomass (BRADSHAW *et al.,* 2000; TAYLOR, 2002). Forests cover about 3.8 billion ha., or 30% of the global land surface. Managed and unmanaged forests throughout the world provide recreational and environmental benefits such as carbon sequestration, renewable energy supplies, watershed protection, improved air quality, biodiversity and habitat for endangered species (Proposal to sequence Populus Genome white paper, http://genome.jgi-psf.org/Poptr1/Poptr1.info.html).

The necessity for model species of plants is well recognized and in this role, *Arabidopsis thaliana* has gained a supreme acceptance amongst plant scientist. While many aspects of tree biology are common to all plants, and hence can be studied in very tractable model species such as *Arabidopsis thaliana*, some unique facets of tree anatomy and physiology must be investigated in trees themselves. The genus *Populus* has been adopted as a model for forest tree genetics. *Populus*, one of only two genera in the family *Salicaceae*, first occurred in the fossil record ca. 60 MYBP. The genus *Populus* is especially well suited to serve as the model genome for trees because of the following reasons: 1) a small genome size – the haploid genome size is ca. 480 ± 20Mbp, 2) rapid juvenile growth, 3) ease of clonal propagation and 4) high-throughput transformation and *in vitro* propagation (BRADSHAW *et al.,* 2000; TAYLOR, 2002; BRUNNER *et al.,* 2004).

A chromosome microdissection and microcloning technology was developed in 1981 (SCALENGHE *et al.,* 1981). Subsequently, it has evolved into an efficient tool for generating chromosome specific DNA libraries of many species (PONELIES *et al.,* 1997; THALHAMMER *et al.,* 2004). This procedure could also be used for the generation of chromosome specific molecular markers, for isolation of chromosome specific sequences using specific PCR primers or for the indirect physical mapping of low/single copy sequences (HOUBEN *et al.,* 2002). With the improvements in the techniques of microdissection and the polymerase chain reaction (PCR), this strategy was simplified largely. Construction of chromosome specific libraries is a potential strategy for the construction of high-density genetic linkage maps of individual chromosomes and the comprehensive analysis of genomes in forest trees. 'Chromosome painting' refers to the hybridization of fluorescently labeled chromosome specific, composite probe pools to cytological preparations. Chromosome painting allows the visualization of individual chromosomes in metaphase or interphase cells and the identification of both numerical and structural chromosomal aberrations with high sensitivity and specificity (RIED *et al.,* 1998). These techniques would also facilitate genomics research in forest trees. However, the application of these techniques to forest trees has seen little reports, largely due to the difficulty of chromosome preparation.

The present study was performed to establish a method for single chromosome microdissection, cloning and painting in forest plants using poplar (*Populus tremula*) as a model. A single chromosome 1 was

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DOI:10.1515/sg-2005-0031
edited by Thünen Institute of Forest Genetics

microdissected from the metaphase spreads of poplar root-tip cells with fine glass needles. The dissected chromosomes were amplified *in vitro* by the *Sau*3A linker adaptor-mediated PCR technique. A microclone library of chromosome 1 was constructed. The amplification products were used as a complex probe mixture and hybridized on the metaphase spreads of poplar.

Materials and Methods

Plant materials

Populus tremula was used as the experimental material in this study. The branches were collected from the plantation in The Research Institute of Forestry, The Chinese Academy of Forestry.

Preparation of mitotic chromosomes

Chromosome samples were prepared by wall degradation hypotonic method according to CHEN *et al.* (1979) with minor modifications. In brief, root tips were removed and immersed successively in saturated paradichlorobenzene for 3 h, in double-distilled water for 30 min, mixed with 2.5% (w/v) cellulase and pectinase (Sigma, Germany) for 30 min, rinsed in double-distilled water for 15 min, and finally fixed in 70% ethanol for 5 min. Put on the fixed material on the sterile coverslip (22×60-mm) and placed drop of 70% ethanol to them, using a microscope slide as a carrier to stabilize the coverslip. Tore the material and removed the supernatant. Added two drops of 70% ethanol on the slide, and then dried with hot air. Twenty slides were produced and counted per tree. Metaphase spreads for FISH were prepared on common microscope slide. The quality of chromosome spreading was evaluated under a microscope (80I, Nikon, Japan). Slides with good quality were used for karyotype analysis, chromosome microdissection and FISH.

Karyotype analysis and identification of chromosome 1

Karyotype analysis was carried out using photographs of clear and well-spreaded metaphase chromosomes according to LI and CHEN (1985). Chromosome 1, the largest chromosome, was identified under a microscope with 1000× magnification (oil objective) based on the result of the karyotype analysis.

Microdissection of chromosome 1

Each air-dried chromosome specimen was immediately used for microdissection. In the chromosome complements prepared by enzymatic maceration, chromosome 1 was unambiguously identified by its size and shape (the largest chromosome). The target chromosome was isolated using an traditional light microscope (BH-2, Olympus, Janpan) equipped with a micromanipulator (MMO-203, Narishige, Japan) and transferred by a fine glass needle pulled by PC-10 puller (Narishige, Japan) into a 0.5 ml tube according to LI *et al.* (1998).

Amplification by Sau3A linker adaptor-mediated PCR amplification (LA-PCR)

The *Sau*3A linker adaptors, with the sequences 5'-CGGGAATTCTGGCTCTGCGACATG-3' and 5'- GATCCATGTC-3' were prepared as described by DENG *et al.* (1992). Isolated chromosomal DNA was treated in 10 µl of 50 ng/µl proteinase K (Merck, Germany) solution at 37°C for 2h. The proteinase K was then inactivated at 65°C for 20 min. The chromosomal DNA was digested by *Sau*3A (0.002U in 1 x T4 ligase buffer, Takara, Japan) at 37°C for 2h. The *Sau*3A was inactivated at 65°C for 20 min, and 20 uM of prepared *Sau*3A linker adaptors and 1U of T4 ligase (Takara, Japan) were added. The ligation between the adaptors and digested chromosomal DNA was performed at 16°C for 16 h. The first round of PCR was carried out in the same tube by adding 10 µl of 10 x Taq buffer, 6 µl of 25 mM $MgCl_2$, 2 µl of 10mM dNTPs, 6 µl of 10 µM 24-mer primer, 2.5 U of Taq DNA polymerase (Takara, Japan), and distilled water to 100 µl. PCR amplifications were performed in a thermal cycler (MG 5331, Eppendorf, Germany) using the following programme: after denaturation at 94°C for 5 min, amplification was performed with 35 cycles of 1 min at 94°C, 1 min at 56°C, and 2.5 min at 72°C, followed by a final extension at 72°C for 10 min. The second round of PCR was carried out using 2 µl of the first-round products as template. The method was the same as described above, except that 20 cycles of amplification were carried out. To monitor possible extraneous DNA contamination, we maintained a negative control (no template DNA) and a positive control (1 pg genomic DNA as template) throughout the whole process.

Southern blot hybridization analysis

The poplar genomic DNA was isolated from leaf tissue using CTAB method according to MURRAY and THOMPSON (1980). DNA molecular weight was checked for quality and quantity by agarose gel (0.8%) electrophoresis and fluorometry (ND-1000, NanoDrop, America). Appropriate amounts of *Eco*R I (Takara, Japan) digested genomic DNA, and the two rounds PCR products from chromosome and controls were separated by electrophoresis (0.8% agarose) and transferred onto nylon membranes (Pall, America). After the poplar genomic probes had been labeled with digoxygenin (DIG)-11-dUTP (Roche, Germany), Southern hybridization and detection were performed following the instructions of the Roche DIG High Prime DNA Labeling and Detection Starter Kit (Roche, Germany).

Construction and characterization of a single chromosome DNA library

The second LA-PCR products from the isolated poplar chromosome 1 were purified using a DNA purification kit (Sangon, China). A 1 µl aliquot of the purified DNA (about 100 ng) was ligated into T-easy vector (Sangon, China) in a 10 µl reaction volume at 16°C for 16 hours. A 1 µl aliquot of the ligation mixture was used to transform *Escherichia coli* strain DH5a competent cells by heat shock. After co-cultivation in luria broth (LB) medium at 37°C for 1 h, one-tenth (v/v) of the cell solution was plated onto LB plates containing 100 ng/µl of ampicillin, 50 ng/µl of X-Gal and 50 ng/µl IPTG for selection of white colonies. 160 randomly selected recombinant plasmids (white colonies) were isolated by alkaline lysis; the insert DNA was amplified using M13 forward/

DOI:10.1515/sg-2005-0031
edited by Thünen Institute of Forest Genetics

reverse primer. The sizes of the inserts were estimated by extrapolating to molecular weight standards on a 1.5% agarose gel. To determine the low/unique or high copy (repetitive sequence) nature of the inserts, DIG-labeled genomic DNA of poplar was hybridized to nylon membranes (Pall, America) containing recombinant clones by dot blotting.

Fluorescence in situ hybridization (FISH)

The second round PCR products were labelled with DIG-dUTP (Roche, Germany) by randomly-primed DNA synthesis, being used as a complex probe mixture for FISH on the metaphase spreads of poplar. FISH was carried out as described by Qi *et al.* (2002). The slides were examined with a Nikon 80i fluorescence microscope (Nikon, Japan). FISH images of suitable metaphases were acquired and stored with a cooled charge-couple device (Spot Diagnostic, America) equipped with the version 4.0.8 Spot Rtke soft (Spot Diagnostic, America).

Results

Chromosome preparation and karyotype analysis

Using root-tip as source material and wall degradation hypotonic method (Chen et al., 1979), we succeeded in preparing good-quality slides of chromosomes in *Populus tremula*. As shown in *Fig. 1A*, chromosomes were spreaded evenly on the slide with a low background. The somatic chromosome number was 2n = 2x = 38. Twenty slides with good quality were used for karyotype analysis. The result was shown in *Fig. 1B*.

Individual chromosome 1 identification and microdissection

The precise identification of microdissected chromosomes was an indispensable prerequisite for the reliability of results obtained in this study. According to the established standard karyotype, chromosome 1, the largest submetacentric chromosome, could be identified at prometaphase or metaphase. After being identified and marked, the target chromosome was successfully isolated by a fine glass needle using an improved chromosome microdissection method in our lab (Li *et al.,* 1998) (*Fig. 2*).

Sau3A linker adaptor-mediated PCR (LA-PCR) amplification of chromosome 1 DNA

In this study, a single chromosome was collected separately into a tube and was used for *Sau*3A LA-PCR. After two sucessive rounds of amplification, the microdissected chromosomes all yielded products that were observed as bright smear electrophoretic bands of DNA with a range from 200bp to 2,500bp on a 1%

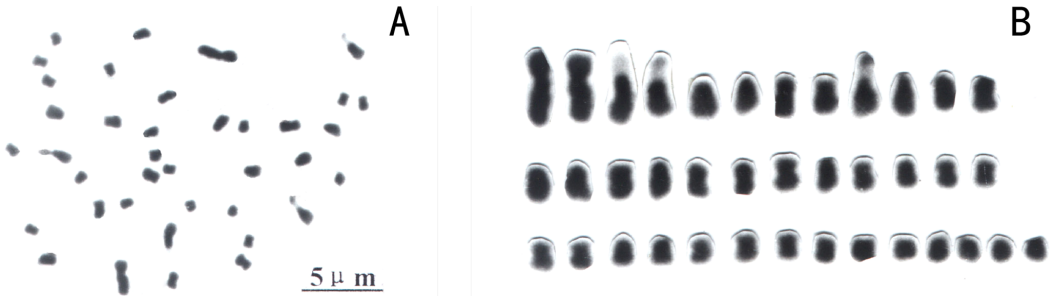

Figure 1. – Image of *Populus tremula* somatic metaphase chromosomes (A) and the karyotype (B), scale bar = 5 μm.

Figure 2. – Procedure of isolation of an individual chromosome 1 in poplar by micromanipulator. A: A Mitotic metaphase image before microdissection of the chromosome 1 (arrow), B: The target chromosome adhering to the tip of a glass needle, C: The individual chromosome 1 in A (arrow) was removed from the cell, scale bar = 5, 10, 5 μm, respectively.

DOI:10.1515/sg-2005-0031
edited by Thünen Institute of Forest Genetics

agarose gel, with most fragments being concentrated between 300bp and 1,200bp (*Fig. 3A*, lane 2). At the same time, the positive control with 1pg of poplar genomic DNA as template for PCR generated products with sizes between 200bp to 4,000bp (*Fig. 3A*, lane 3). As a negative control for monitoring possible contamination with DNA, a sample without template DNA was set up during all stages of the microdissection and amplification procedures. None of the products was amplified from the negative control (*Fig. 3A*, lane 1). The results indicate that DNA from chromosome 1 was amplified successfully without contamination of exogenous DNA. Southern hybridization with DIG-labeled genomic DNA confirmed that the products were amplified from the poplar genome (*Fig. 3B*).

Construction of the chromosome 1 specific DNA library

The second round LA-PCR products from the single chromosomes 1 were used to prepare ligation mixtures for transformation. Approximately 3 x 10^5 recombinant clones (white colonies) were obtained. 160 randomly selected white colonies were selected for further analysis

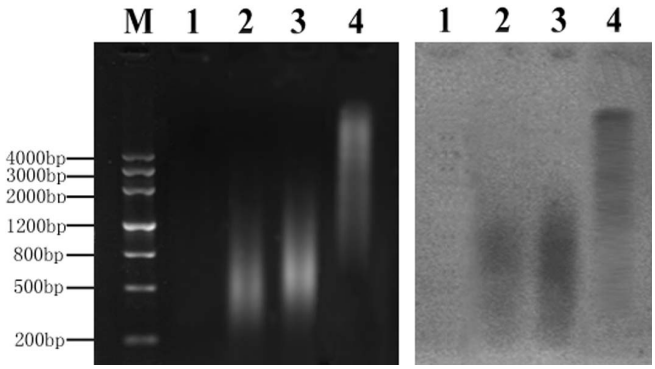

Figure 3. – Linker adaptor-mediated polymerase chain reaction (LA-PCR) amplification with microdissected chromosomes 1 and southern blot hybridization analysis. Left: The LA-PCR products of microdissected chromosomes: the DNA molecular-weight mark lane M, the negative control (lane 1), the single chromosome 1 as DNA template (lane 2), the positive control (lane 3), *EcoR*I-digested genomic poplar DNA (lane, 4). Right: Southern blot hybridization of the PCR products with DIG-labeled genomic poplar DNA.

The length of the inserts ranged mainly from 230 to 2,200 bp, with an average of 800 bp, as estimated by 1.5% agarose gel electrophoresis (*Fig. 4*). The colony microarray hybridization analysis were carried out to estimate the copy number of the inserts. Of the 160 clones examined, 40% represented low/unique copy sequences, while 60% of the clones were medium or highly repetitive sequences (*Fig. 5*).

Chromosome painting of chromosome 1

The LA-PCR products originating from individual microdissected chromosome 1 were labeled with DIG-dUTP, and hybridized to mitotic metaphase spreads in the absence of a competitor. Signals were mainly observed uniformly in all chromosomes 1 (*Fig. 6A*). At the same time, some signals were also observed on the terminal and centromeric regions of other chromosomes (*Fig. 6A*). Then, unlabeled shared total DNA of poplar was added as a competitor. FISH experiments, which included different probe/competitor combinations, were performed. In all cases, the pattern of signals distributing had not greatly change, although weaker than without pre-hybridization with competitor DNA (even competitor DNA in 100-fold and excess for a 2 h pre-hybridization) (data not shown).

Discussion

Preparation of mitotic chromosomes

A good-quality chromosome spread was essential in this method and was technically demanding to perform.

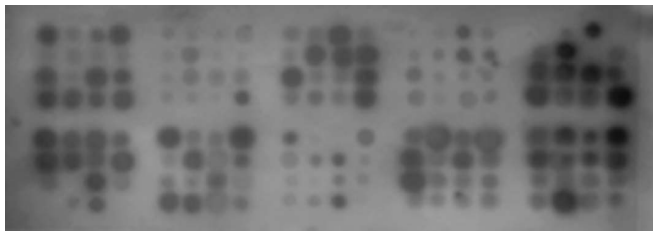

Figure 5. – Result of bacterial colonies hybridization. The 160 randomly selected recombinant clones were positioned on a membrane in a grid microarray. The colony microarray was hybridized with a DIG-labeled genomic poplar DNA probe.

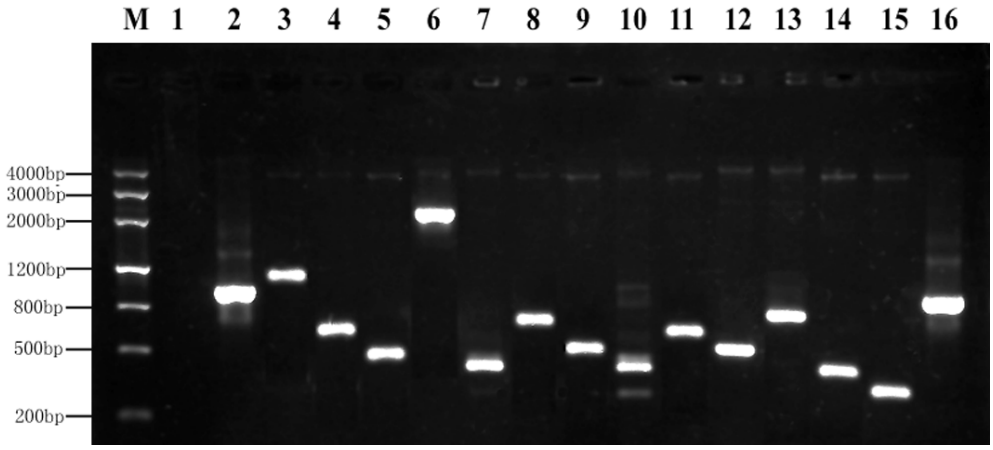

Figure 4. – Partly electrophoresis result of ampified cloning fragments by PCR using M13 forward/reverse primers.

DOI:10.1515/sg-2005-0031
edited by Thünen Institute of Forest Genetics

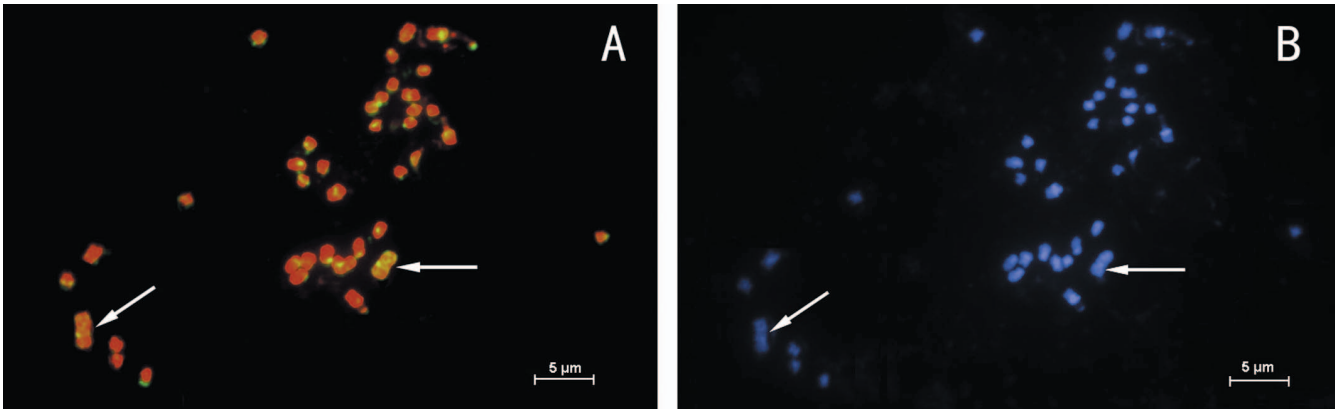

Figure 6. – Result of chromosome painting using microdissection amplification products as prob. A: Metaphase spread of poplar hybridized with labeled LA-PCR products of chromosome 1 without blocking DNA. B: The same chromosomes as shown in A after staining with 4',6-diamidino-2-phenylindole. Arrows indicate the chromosome 1, scale bar = 5 µm.

This step may be a bottleneck hindering this type of research in poplar because they usually have thick cell walls and were highly lignified, making chromosome spreading difficult. Using root-tip as source material and the wall degradation hypotonic method according to CHEN *et al.* (1979) with minor modifications, we succeeded in preparing high quality slides of chromosomes of *Populus tremula*.

The period of chromosome fixation in acetic acid should be as short as possible, because acetic acid damages DNA by depurination. An extended period of fixation in acetic acid could result in relatively short (50–100 bp long) chromosomal DNA fragments (HOUBEN *et al.,* 2002). Therefore, we avoided fixing root-tip in fixation solution (methanol : acetic acid = 3:1) and used 70% ethanol for short time fixation in chromosome preparation. The chromosome spreads were then prepared in 70% ethanol instead of 45% acetic acid. These steps avoided acid depurination and mechanical damage of chromosomal DNA.

Identification and microdissection of target chromosomes

Correct identification of the target chromosomes is also an important step required for single chromosome microdissection. Generally, this is achieved by choosing plants with target chromosomes bearing prominent morphological features, such as the largest or smallest chromosomes (FUKUI *et al.,* 1992; CHEN and ARMSTRONG, 1995; STEIN *et al.,* 1998; HUANG *et al.,* 2004), or by using specific plant species, for example, monosomic lines (JUNG *et al.,* 1992; VEGA *et al.,* 1994), or tritelosomic lines (SCHONDELMAIER *et al.,* 1993). In *Populus tremula*, the chromosome 1, the largest submetacentric chromosome, could be unambiguously identified at prometaphase or metaphase. Only metaphases with unequivocally identifiable and spatially well-separated chromosomes were used for microdissection.

However, because of the small sizes of chromosomes in poplar, it is difficult to identify the target chromosome correctly under a 40 x objective. In this paper, we used a technique that was improved by our lab. (LI *et al.,* 1998) for the precise chromosome microdissection by ordinary light microscope. It had not only solved the difficulty of chromosome microdissection by oil objective, but achieved the microdissection on high magnifying multiple (magnification 1,000x~1,500x), and greatly improved the accuracy of chromosome microdissection.

Generally, the sterility requirements to prevent contaminations have to be the same in PCR as for tissue culture. Exceptional care must be taken in preparing reagents for chromosome microdissection to avoid contaminant DNA being amplified (HOUBEN *et al.,* 2002). Each working solution is prepared, divided into single-use aliquots, tested, and used for microdissection only if satisfactory. When preparing solutions, wear gloves and, to the extent it is possible, conduct all manipulations in a laminar flow hood. Irradiate plastic and glassware, buffers and stock solutions (except nucleotides, primers, linker-adaptors and enzymes) with UV light (260 nm wavelength) for 12 hours.

Construction of chromosome specific DNA library

In this paper, We have successfully constructed a plasmid library from a single microdissected poplar chromosome 1 by used LA-PCR. In previous studies, PCR-mediated cloning was usually performed based on DNA templates provided by several isolated target chromosomes (HOUBEN *et al.,* 2002). However, microdissection of specific chromosomes as small as those in poplar is complicated and elaborate work, requiring skill and experience, hence it is impracticable to isolate many copies of the same chromosome from different metaphases. To tackle this problem, we tried to acquire DNA fragments by conducting PCR based on a single copy of a target chromosome template. Conditions for amplification of chromosomal DNA were optimized to detect and amplify DNA between 50–100 femtogram. Our results indicate it is feasible to amplify DNA fragments from a single chromosome with small size for constructing the chromosome specific DNA library.

To date, the cloning efficiency of PCR products from microdissected plant chromosomes has ranged from 2 x 10^4 (JUNG *et al.,* 1992) to 5 x 10^5 (CHEN and ARMSTRONG, 1995), and then to 4 x 10^6 (STEIN *et al.,* 1998). All members of the *Populus* genus have a genome contained 19 nearly identical, metacentric chromosomes, a nuclear

DOI:10.1515/sg-2005-0031
edited by Thünen Institute of Forest Genetics

content of 2C = 1.2 pg and the haploid genome size of poplar is only ca. 480 Mbp (BRADSHAW *et al.,* 2000; TAYLOR, 2002). Since chromosome 1 covers about 5% of the genome per haploid (ZHANG *et al.,* 2005), it is deduced to contain 0.06 pg or approximately 24Mbp. The microclone library of chromosome 1, in this study, included 3 x 10^5 clones, with an average insert size of 800 bp. For a 99% probability that every sequence of chromosome 1 is represented in at least one recombinant clone, 1.4 x 10^5 clones of chromosome 1 are needed (SAMBROOK *et al.,* 1989). So this library maybe can represent the whole of chromosome 1 of poplar, in the main. However, when linker adapter is used for fragment of one restriction enzyme, theoretically there is a possibility of causing bias. Especially the case of highly repetitive sequences the bias became larger. So, till now, none of the chromosome microclone libraries reported could reach a 100% coverage rate. The way to obtain a genomic library that covers a whole target chromosome would be to employ different combinations of restriction enzymes, adaptors or primers to generate libraries with different complements of DNA sequences (JUNG *et al.,* 1992).

The quality of the chromosome library, on the other hand, is influenced by the enrichment of unique/low copy or chromosome specific sequences. The poplar nuclear genome contains approximately 60%–80% repetitive DNA sequences (FLAVELL *et al.,* 1974, 1980; HOUBEN *et al.,* 2002). In the library of chromosome 1, the frequency of repetitive inserts was 60%, while that of low/single copy sequences was 40%. Similar results were obtained by CHEN and ARMSTRONG (1995), LIU *et al.* (1997), STEIN *et al.* (1998) and ZHOU *et al.* (1999).

Feasibility of chromosome painting

The concept of chromosome painting was first introduced in 1988 (LICHTER *et al.,* 1988; PINKEL *et al.,* 1988). Chromosome painting has over the last few years become an established procedure in laboratories working with mammalian chromosomes (ANTONACCI *et al.,* 1995). To ensure specific hybridization to related chromosome segments, repetitive sequences need to be excluded from the hybridization process by, for example, pre-hybridization with a large excess of unlabelled total genomic DNA or the fast-reassociating (Cot-1) fraction of genomic DNA (HOUBEN *et al.,* 2002).

However, chromosome painting in plants is relatively underdeveloped, although this technique would be useful for evolutionary studies and plant breeding. A comprehensive study by FUCHS *et al.* (1996) outlines past endeavors to paint chromosomes from various plant species with large genomes (2C DNA content of 11 to 40 pg, such as barley, wheat, *Vicia faba*, *Picea abies*). No specific painting of the chromosomes was obtained although a number of different approaches, including pre-hybridization with a large excess of total unlabelled genomic DNA, were tested.

Successful chromosome painting in plants with relatively large genomes has been reported for B chromosomes of *Secale cereale* (HOUBEN *et al.,* 1996), *Brachycome dichromosomatica* (HOUBEN *et al.,* 1997) and the Y chromosome of *Rumex acetosa* (SHIBATA *et al.,* 1999). However, the painting of the described B or Y chromosome was possible because of enrichment for chromosome specific repetitive sequences, rather than the chromosome-specific low- and single-copy sequences which are responsible for painting mammalian chromosomes (HOUBEN *et al.,* 2002).

These results were similar to our present study. Hybridization with or without competition by unlabeled genomic DNA showed signals on all the metaphase chromosomes. Our painting pattern indicated that the amplification products of chromosome probably failed to specially label chromosome 1 owing to the presence of a large number of chromosome 1 nonspecific repetitive sequences, which were dispersed at high frequency in the genome. This conclusion accorded with component of chromosome 1 specific DNA library, constructing in this study, in which frequency of repetitive inserts was 60%, while that of low/single copy sequences was 40%. So, we could consider that Chromosome 1 contain large amounts of chromosome specific, repetitive DNA sequences and dispersed repetitive DNA sequences, common to all chromosomes.

Without doubt, with the method described here, the high resolution obtainable by the microdissection of poplar chromosomes could provide a valuable tool for constructing a genetic map, the physical mapping of chromosomes and the isolation of useful genes, as well as improving genetic mapping and comparative genomic researches in poplar.

Acknowledgements

The authors thank Ms. DULIAN QIU from Sichuan Academy of Agricultural Sciences of China for helpful suggestions on the manuscript. Financial support for this study is from National Key Basic Research Program ("973") (G19990160) – "Molecular Research on Trees Improvement".

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DOI:10.1515/sg-2005-0031
edited by Thünen Institute of Forest Genetics

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DOI:10.1515/sg-2005-0031
edited by Thünen Institute of Forest Genetics