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ORIGINAL PAPER

D. Huang · W. Wu · Y. Zhou · Z. Hu · L. Lu

Microdissection and molecular manipulation of single chromosomes in woody fruit trees with small chromosomes using pomelo (*Citrus grandis*) as a model. I. Construction of single chromosomal DNA libraries

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Abstract Construction of single chromosomal DNA libraries by means of chromosome microdissection and microcloning will be useful for genomic research, especially for those species that have not been extensively studied genetically. Application of the technology of microdissection and microcloning to woody fruit plants has not been reported hitherto, largely due to the generally small sizes of metaphase chromosomes and the difficulty of chromosome preparation. The present study was performed to establish a method for single chromosome microdissection and microcloning in woody fruit species using pomelo as a model. The standard karyotype of a pomelo cultivar (*Citrus grandis* cv. Guanxi) was established based on 20 prometaphase photomicrographs. According to the standard karyotype, chromosome 1 was identified and isolated with fine glass microneedles controlled by a micromanipulator. DNA fragments ranging from 0.3 kb to 2 kb were acquired from the isolated single chromosome 1 via two rounds of PCR mediated by *Sau*3A linker adaptors and then cloned into T-easy vectors to generate a DNA library of chromosome 1. Approximately 30,000 recombinant clones were obtained. Evaluation based on 108 randomly selected clones showed that the sizes of the cloned inserts varied from 0.5 kb to 1.5 kb with an average of 860 bp. Our research suggests that microdissection and microcloning of single small chromosomes in woody plants is feasible.

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D. Huang · L. Lu (✉)
College of horticulture,
Fujian Agriculture and Forestry University,
350002 Fuzhou, P.R. China
e-mail: zjweiren@pub5.fz.fj.cn
Tel.: +86-591-3789176
Fax: +86-591-3789176

D. Huang
College of Bio-engineering,
Fujian Normal University,
350007 Fuzhou, P.R. China

W. Wu
Department of Agronomy,
College of Agriculture and Biotechnology,
Zhejiang University,
310027 Hangzhou, P.R. China

W. Wu
College of Crop Science,
Fujian Agriculture and Forestry University,
350002 Fuzhou, P.R. China

Y. Zhou · Z. Hu
Institute of Genetics and Developmental Biology,
Chinese Academy of Sciences,
100101 Beijing, P.R. China

Introduction

Fruit trees are important economical crops. However, genetic studies in fruit trees have proved to be difficult relative to other crop species such as cereals and vegetables owing to their large size, long juvenile period, high heterozygosity and, in some species, even self-incompatibility. Although molecular marker linkage maps have been constructed in a few fruit tree species in recent years, the maps are not dense enough for the purpose of mapping and cloning genes or quantitative trait loci (QTLs) controlling important agronomical characters.

Chromosome microdissection is an attractive technology in genome research because unlimited copies of DNA fragments representing the isolated chromosome can be obtained. These DNA fragments can be used as probes for chromosome painting. They can also be cloned to generate a chromosome-specific DNA library, which would be useful for positional cloning of genes located in the chromosome. Since the first successful cloning of specific chromosome regions in polytene chromosomes of *Drosophila* was reported in 1981 (Scalenghe et al. 1981), chromosome microdissection and microcloning has been developed into an efficient tool for producing mini-DNA libraries of specific single chromosomes or chromosomal

segments. To date, chromosome-specific libraries have been successfully created in human, animals and several plant species such as wild beet (Jung et al. 1992), barley (Schondelmaier et al. 1993), wheat (Albani et al. 1993; Vega et al. 1994; Liu et al. 1997), oat (Chen and Armstrong 1995), maize (Stein et al. 1998) and rye (Houben et al. 1996; Zhou et al. 1999). A few clones from these libraries have been utilized for probing RFLP (restriction fragment length polymorphism; Chen and Armstrong, 1995) and tagging important genes (Jung et al. 1992; Schondelmaier et al. 1993).

This technology would also facilitate genomics research in fruit trees. 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 fruit trees. However, application of the technology to woody fruit plants has not been reported, largely due to the difficulty of chromosome preparation and generally small sizes of metaphase chromosomes. The study reported here was an attempt to initiate this type of work in woody fruit trees. Pomelo is an important subtropical woody fruit plant found in Southern China. We chose pomelo as a model to establish a technical system of single-chromosome microdissection and microcloning in fruit tree species with small chromosomes.

Materials and methods

Plant material

Pomelo var. *Guanximiyou* (*Citrus grandis* cv. Guanxi) was used. Pomelo is a diploid species containing 18 chromosomes, which are typically small (0.5~2 μm) in mitotic metaphase.

Chromosome preparation

Young pomelo embryos approximately 1–2 mm in size were collected about 100 days after pollination, fixed in ethanol-acetic acid (3:1) for 2 min, and then stored in 70% ethanol at 4°C. Following hypotonic treatment in ddH_2O for 20 min, several embryos were digested in 200 μl enzyme solution (2.5% cellulase Onozuka R-10, 2.5% Pectolyase Yakul Y-23 in ddH_2O) at 37°C for 45 min. After careful rinsing, the embryos were ground with a pipette in about 30 μl improved Carbol Fuchsin stain solution. A drop of the stained material in suspension was placed on a glass slide and squashed. The quality of chromosome spreading was evaluated under a microscope (ZEISS, AXIOLAB). Slides of good quality were used for karyotype analysis and chromosome microdissection.

Karyotype analysis and identification of chromosome 1

Karyotype analysis was carried out using photographs of clear and well-spread metaphase or prometaphase chromosomes according to Levan et al. (1964). Chromosome 1 (the largest chromosome) was identified under a microscope with 100× magnification (10×100, oil immersion) based on the result of the karyotype analysis.

Microdissection of target chromosome

The cover slip was removed after the slide was soaked in liquid nitrogen for 2 min. The slide was then air-dried and used for microdissection immediately. Glass needles with a fine tip (0.2~0.5 μm in diameter) were constructed with a needle puller and an alcohol burner. The target chromosome was isolated under an inverted microscope (Nikon, TMS-F; 15×40) using a glass needle controlled by a micromanipulator (NT-188NE) and transferred into a microcentrifuge tube containing 20 μl proteinase K solution (5 ng/μl in 1× T4 ligase buffer; Promega, Madison, Wis.).

*Sau*3A linker adaptor-mediated PCR amplification (LAM-PCR)

*Sau*3A linker adaptors with complementary sequences 5′-GATC-CTGAGCTCGAATTCGAC CC-3′ and 3′-GACTCGAGCT-TAAGCTGGG-5′ were prepared according to Chen and Armstrong (1995). The isolated chromosome was incubated in the proteinase K solution at 37°C for 3 h to remove proteins and then transferred to 75°C for 20 min to inactivate the proteinase K. The naked chromosomal DNA was further digested by *Sau*3A (0.02 U in 1× T4 ligase buffer; Promega) at 37°C for 4 h and then incubated at 70°C for 20 min to inactivate the *Sau*3A. Subsequently, 10 ng of *Sau*3A linker adaptor and 1.5 U T_4 ligase (Promega) were added to the same tube. The ligation between the adaptor and digested chromosomal DNA was performed at 16°C for 16 h or overnight. At the end of the incubation period, the T_4 ligase was inactivated at 70°C for 20 min. Two rounds of PCR were conducted to amplify the DNA fragments of the isolated chromosome. The first round was performed in the same tube by adding 10 μl 10× *Taq* buffer, 6 μl 25 m*M* $MgCl_2$, 2 μl 10 m*M* dNTPs, 250 ng 19-mer primer, 2.5 U *Taq* DNA polymerase (Promega) and distilled water to a final volume of 100 μl. After a denaturation at 94°C for 5 min, amplification was performed with 35 cycles of 1 min at 94°C, 1.5 min at 50°C and 2 min at 72°C, followed by a final extension at 72°C for 15 min. The second round of PCR was carried out using 2 μl of the first-round products as template and following the same procedure as the first round except that only 20 amplification cycles were conducted. To monitor possible extraneous DNA contamination, we maintained a negative control (no template DNA) and a positive control (10 pg pomelo genomic DNA as template) throughout the whole process.

Southern hybridization

Genomic DNA was extracted from young leaves using the CTAB method (Kidwell and Osborn 1992) and digested with *Eco*RI. The digested genomic DNA and the LAM-PCR products from the isolated chromosome were electrophoresed on a 0.8% agarose gel at 40 ′v for approximately 6–8 h and then transferred onto nylon membranes (Minipore). Southern hybridization and detection with genomic DNA as a probe (labeled with DIG-11-dUTP by the random primed DNA labeling method) was performed according to the instructions provided with the DIG DNA Labeling and Detection kit (Boehringer, Mannheim, Germany).

Library construction

The LAM-PCR products from the isolated chromosome were purified using a DNA purification kit (Shanghai Sangon). A 2-μl aliquot of the purified DNA was ligated into T-easy vector (Promega) in a 10-μl reaction volume at 4°C for 16 h or overnight. A 1-μl aliquot of the ligation mixture was used to transform DH5α competent cells by heat shock. After co-cultivation in SOC medium (2% bactotryptone, 0.5% yeast extract, 10 m*M* NaCl, 2.5 m*M* KCl, 10 m*M* $MgCl_2$, 10 m*M* $MgSO_4$, 20 m*M* glucose) at 37°C for 1 h, one-tenth (v/v) of the cell solution was spread on an ampicillin (100 g/l)-IPTG-x-gal plate to screen for transformed cells.

Analysis of plasmid clones

Recombinant plasmid DNAs were isolated from 108 randomly selected white colonies by the alkaline minipreparation method. Insert DNAs were released by digestion with *Eco*RI or by PCR amplification using T7/SP6 primers. The sizes of the inserts were estimated by extrapolating to molecular-weight standards on a 1.4% agarose gel.

Results

Chromosome preparation and karyotype analysis

Using young embryos as source material and the methods of enzymatic maceration and squashing, we succeeded in preparing good-quality slides of chromosomes of *Citrus grandis* cv. Guanxi. As shown in Fig. 1a, chromosomes were spread evenly on the slide with a clear background. The somatic chromosome number was $2n=2x=18$ as expected. However, different chromosomes were difficult to distinguish at the mitotic metaphase because of their small sizes and morphological similarities. Comparatively, chromosomes were more distinguishable at prometaphase than at metaphase. For this reason, we selected 20 photos of prometaphase chromosomes instead of metaphase ones for karyotype analysis. The result is shown in Fig. 1b.

Identification and microdissection of chromosome 1

According to the established standard karyotype, chromosome 1, the largest submetacentric chromosome, could be identified at prometaphase or metaphase. Although chromosomes 1 and 2 are similar in length, they could be distinguished under the oil immersion objective (100×) because chromosome 2 was metacentric. After being identified and marked, chromosome 1 was successfully isolated by a fine glass needle (Fig. 2).

*Sau*3A linker adaptor-mediated PCR amplification

After two successive rounds of amplification, no product was amplified in the negative control (Fig. 3, lane 1), while the isolated chromosome yielded products with sizes ranging between 300 bp and 2,000 bp with the majority in the range of 500 bp to 1,500 bp (Fig. 3, lane 2) and the positive control generated products of sizes between 300 bp and 3,000 bp (Fig. 3, lane 3). 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 pomelo genome.

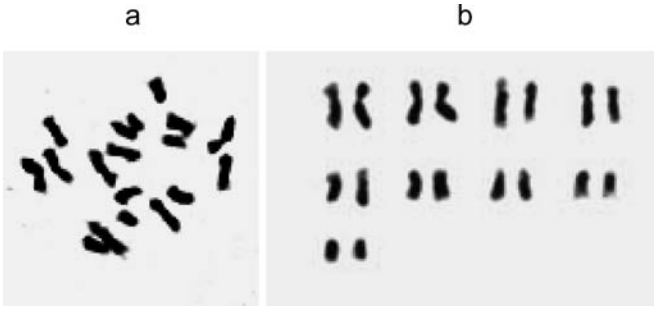

Fig. 1 Image of somatic metaphase chromosomes at 1,200× magnification (**a**) and the karyotype (**b**)

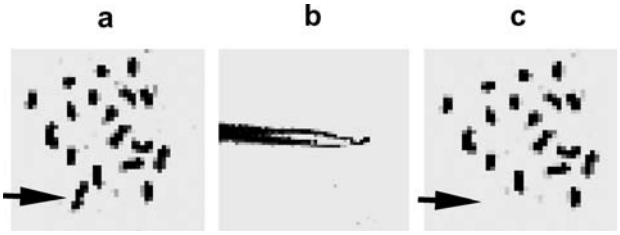

Fig. 2a–c Procedure of microdissection of chromosome 1 in pomelo under an inverted microscope at 600× magnification. **a** Mitotic metaphase image before the target chromosome (*arrow*) was isolated. **b** The target chromosome adhering to the tip of a glass needle. **c** Mitotic metaphase image after the target chromosome was isolated

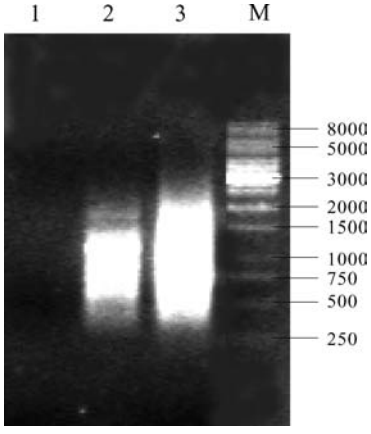

Fig. 3 Results of second-round LAM-PCR. *Lanes*: *1* Negative control, *2* single chromosome of pomelo, *3* positive control, *M* 1-kb DNA ladder

Construction of single chromosome library

The LAM-PCR products from the second-round amplification of the isolated chromosome 1 were used to prepare ligation mixtures for transformation. Approximately 120–180 white colonies were obtained each time when a small fraction (1/200) of the second-round PCR products were used. Extrapolation suggested that there were about 3×10^4 colonies in this chromosomal DNA library. Plasmid DNAs were prepared from 108 randomly selected white colonies. The lengths of the inserts, as compared with known molecular markers run on a 1.4% agarose gel

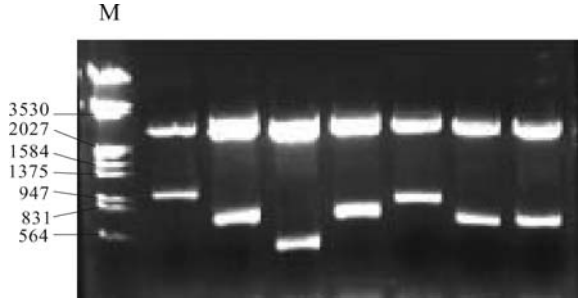

Fig. 4 Result of enzymatic digestion of plasmid DNAs of several recombinant clones. *M Eco*RI/*Hin*dIII-digested λDNA

(Fig. 4), ranged from approximately 300 bp to 1,800 bp, with an average of 860 bp; 92 (88%) of the inserts had a length greater than 500 bp.

Discussion

Preparation of mitotic chromosomes

Preparation of good-quality chromosomes is the first step in the construction of single-chromosome DNA libraries. This step may be a bottleneck hindering this type of research with woody plants because they usually have thick cell walls and are highly lignified, making chromosome spreading difficult. In order to make good chromosome spreads suitable for karyotype analysis (this may be unnecessary if the karyotype is known, but for pomelo, no study on karyotype analysis has been reported) and chromosome microdissection, two factors should be considered.

The first is the plant tissue used. In general, root tips are most frequently used for chromosome preparation. In pomelo (and perhaps also in other woody plant species), however, we have found that young embryos are more appropriate for chromosome preparation. In comparison with root tips, young embryo cells have a thinner cytoplasm and, consequently, better chromosome spreading can be obtained. In addition, young embryos have a higher cell division index. We acquired many metaphase and prometaphase chromosomes without pretreatment to synchronize cell division. This simplifies the procedure for chromosome preparation and, more important, reduces the use of chemical reagents and thus decreases the possible lesions in DNA molecules caused by such chemicals. Intact DNA molecules are required for the microcloning of single chromosomes.

The second factor to be considered is the methodology. The most important factor here is that lesions (such as depurination) in DNA molecules should be avoided by all means. With this in mind, we adopted a very short fixation time (<3 min) for young embryos in Carnoy solution and used enzymes (instead of HCl or other chemicals) to macerate cell walls for chromosome preparation. Our study has proven that these methods work well.

Identification of target chromosomes

Correct identification of the target chromosomes is also an important step required for single-chromosome microdissection and microcloning. In some studies, identification of target chromosomes was achieved by utilizing special plant materials such as monosomic lines (Jung et al. 1992; Vega et al. 1994) and tri-telosomic lines (Schondelmaier et al. 1993). In other studies in which special materials were not available, only those chromosomes with apparent morphological features, such as the largest or the smallest ones, were selected as the targets (Fukui et al. 1992; Chen and Armstrong 1995; Stein et al. 1998). In the present study, we also targeted an individual chromosome initially characterized by karyotype analysis. However, because of the small sizes of chromosomes in pomelo, it is difficult to identify the target chromosome correctly under a 40× objective. To avoid mistakes, we identified the target chromosome under an oil immersion (100×) objective, drew a draft picture of the mitotic image and marked the target chromosome on a piece of paper before isolating it under the 40× objective. Microdissection of specific chromosomes as small as those in pomelo is complicated and elaborate work, requiring skill and experience. However, even for a skillful and experienced person, it is still not possible to distinguish all of the chromosomes on the basis of their cytological appearances. To solve this problem, molecular biological technologies might be useful. We may be able to isolate individual chromosomes randomly, amplify them separately in vitro and then identify them by hybridizing certain probes (such as cDNA clones from the same species) with their PCR products. If this approach works, it would be possible to microdissect all of the chromosomes in a genome without much difficulty, and the technology of chromosome microdissection will be applicable to most, if not all, plant species no matter what their chromosome sizes.

Construction of a chromosomal DNA library by amplification of a single chromosome

At present, there are two distinct approaches to construct DNA libraries from microdissected chromosomal DNA in plants. One is direct cloning (Sandery et al. 1991), for which a large number of isolated target chromosomes is required to provide sufficient DNA molecules. The other approach is PCR-mediated cloning (Jung et al. 1992; Schondelmaier et al. 1993; Telenius et al. 1992; Stein et al. 1998), which requires many fewer isolated target chromosomes. According to the primers used, the method of PCR-mediated cloning can be further divided into two types: vector/adaptor-mediated PCR (i.e. LAM-PCR; Jung et al. 1992, Schondelmaier et al. 1993; Chen and

Armstrong 1995), which uses specific primers, and degenerate oligonucleotide primed PCR (DOP-PCR; Telenius et al. 1992; Pich et al. 1994; Liu et al. 1997), which uses random primers. To date, both PCR-based techniques have become important methods for creating chromosome-specific libraries in plants. Studies have shown that LAM-PCR usually produces larger DNA fragments than DOP-PCR (Zhou et al. 2000). As the size of the inserted DNA fragments is a key to the quality of microclone libraries (Chen and Armstrong 1995; Zimmer et al. 1997) and the optimal sizes of probes for RFLP analysis are usually suggested to be greater than 300 bp, we used the method of LAM-PCR in this study—and we did obtain amplified fragments with sizes ranging from 300 bp to 2,000 bp as hoped.

In previous studies, PCR-mediated microcloning was usually performed based on DNA templates provided by several isolated target chromosomes. However, most chromosomes in woody fruit plants are very small and indistinguishable under a microscope, and 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. Our results indicate that amplifying DNA fragments from a single chromosome of small size for constructing the chromosome-specific DNA library is feasible. To date, there are only a few reports on the construction of DNA libraries from single chromosomes (Chen and Armstrong, 1995; Dang et al. 1998), and there is no such report in woody fruit plants. Therefore, the present study represents a significant advance in chromosome microdissection and microcloning in fruit trees.

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References

Albani D, Cote MJ, Armstrong KC, Chen Q, Segal A, Robert LS (1993) PCR amplification of microdissected wheat chromosome arms in a simple single tube reaction. Plant J 4:899–903

Chen Q, Armstrong K (1995) Characterization of a library from a single microdissected oat (*Avena sativa* L.) chromosome. Genome 38:706–714

Dang BY, Hu ZM, Zhou YH, Cui LH, Wang LL, Li LC, Chen ZH (1998) Construction of single-chromosome DNA library from *Lilium regal*e Wilson. Chin Sci Bull 43:434–438

Fukui K, Minezawa M, Kamisugi K, Ishikawa M, Ohmido N, Yanagisawa T, Fujishita M, Sakai F (1992) Microdissection of plant chromosomes by argon-ion laser beam. Theor Appl Genet 84:787–791

Houben A, Kynast RG, Heim U, Hermann H, Jones RN, Forster JW (1996) Molecular cytogenetic characterisation of the terminal heterochromatic segment of the B-chromosome of rye (*Secale cereale*). Chromosoma 105:97–103

Jung C, Claussen U, Horsthemke B, Fischer F, Herrmann RG (1992) A DNA library from an individual *Beta patellaris* chromosome conferring nematode resistance obtained by microdissection of meiotic metaphase chromosome. Plant Mol Biol 20:503–511

Kidwell KK, Osborn TC (1992) Simple plant DNA isolation procedures. In: Beckmann JS, Osborn TC (eds) Plant genomes: methods for genetic and physical mapping. Kluwer, Dordrecht, pp 1–13

Levan A, Fredga K, Sondberg AA (1964) Nomenclature for centromeric position of chromosomes. Hereditas 52:201–220

Liu B, Segal G, Vega JM, Feldman M, Abbo S (1997) Isolation and characterization of chromosome-specific DNA sequences from a chromosome arm genomic library of common wheat. Plant J 11:959–965

Pich U, Houben A, Fuchs J, Meister A, Schubert I (1994) Utility of DNA amplified by degenerate oligonucleotide-primed PCR (DOP-PCR) from the total genome and defined chromosomal regions of field bean. Mol Gen Genet 243:173–177

Sandery MJ, Forster J, Macadam SR, Blunden R, Jones RN, Brown SDM (1991) Isolation of a sequence common to A- and B-chromosomes of rye (*Secale cereale*) by microcloning. Plant Mol Biol Rep 9:21–30

Scalenghe F, Turco E, Edstrom JE, Pirrotta V, Melli MI (1981) Microdissection and cloning of DNA from a specific region of *Drosophila melanogaster* polytene chromosomes. Chromosoma 82:205–216

Schondelmaier J, Martin R, Jiahoor A, Houben A, Graner A, Koop HU, Herrmann RG, Jung C (1993) Microdissection and microcloning of the barley (*Hordeum vulgare* L.) chromosome 1HS. Theor Appl Genet 86:629–636

Stein N, Ponelies N, Musket T, McMullen M, Weber G (1998) Chromosome microdissection and region-specific libraries from pachytene chromosomes of maize (*Zea mays* L.). Plant J 13:281–289

Telenius H, Carter NP, Bebb CE, Nordenskjold M, Ponder BAJ, Tunnacliffe A (1992) Degenerate oligonucleotide-primed PCR: general amplification of target DNA by a single degenerate primer. Genomics 13:718–725

Vega JM, Abbo S, Feldman M, Levy AA (1994) Chromosome painting in plants: in situ hybridization with a DNA probe from a specific microdissected chromosome arm of common wheat. Proc Natl Acad Sci USA 91:12041–12045

Zhou YH, Hu ZM, Dang BY, Wang H, Deng XD, Wang LL, Chen ZH (1999) Microdissection and microcloning of rye (*Secale cereale* L.) chromosome 1R. Chromosoma 108:250–255

Zhou YH, Wang H, Wei J et al. (2000) Comparison of two PCR techniques used in amplification of microdissected plant chromosomes from rice and wheat. Biotechniques 28:766–774

Zimmer R, Haberfeld A, Gebbins AMV (1997) Microisolation of the chicken Z chromosome and construction of microclone libraries. Genome 40:865–872