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Microdissection and painting of the W chromosome in *Ginkgo biloba* showed different labelling patterns

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ABSTRACT. The chromosome 1 with bigger satellite, supposed to be the W chromosome, was microdissected from the metaphase spreads of female ginkgo root-tip cells with a 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. Southern hybridization analysis indicated that DNA from the single W chromosome was successfully amplified. FISH analyses with the PCR products have been performed in the metaphase spreads of female and male ginkgo. FISH signals were observed along the entire W chromosome, while along about 1/2 length at the end of the long arm of the Z chromosome, the intensity of signals was lower than at other segments. This suggested that the chromosome 1 of ginkgo might harbor different DNA sequence structures at the 1/2 length end of the long arm, and this region on the W chromosome might be a female-specific region which formed during evolution of the sex chromosomes.

Keywords: Chromosome painting; FISH; *Ginkgo biloba*; Microdissection; Sex chromosome.

INTRODUCTION

Ginkgo biloba is a dioecious gymnosperm species with both male and female plants having $2n = 2x = 24$ chromosomes, consisting of four metacentrics and twenty subtelocentrics. Several studies on the sex determination system and sex chromosomes of ginkgo have appeared since the 1950s. Newcomer (1954) and Chen et al. (1987) both reported the ZW-type sex chromosome system might be present in ginkgo. Chen et al. (1987) found that the size of the Ag-NOR on chromosome 1 from females is different while it is the same from the male. So far, however, no further study on the chromosome 1 has been reported.

The direct strategy for isolating sequences from chromosomes of interest is to separate them by a flow-sorting procedure, or by microdissection. A chromosome microdissection 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).

Chromosome painting refers to the hybridization of fluorescently-labeled, chromosome specific, composite probe pools to cytological and structural chromosomal aberrations with high sensitivity and specificity (Ried et al., 1998). The concept of chromosome painting was first introduced in 1988 (Lichter et al., 1988; Pinkel et al., 1988). It has over the last few years become an established procedure in laboratories working with mammalian chromosomes (Antonacci et al., 1995). In plants, however, chromosome painting is relatively underdeveloped. In plants, to ensure specific hybridization to related chromosome segments, repetitive sequences need to be excluded from the hybridization process by, for example, blocking with a large excess of unlabelled total genomic DNA or the Cot-1 fraction of genomic DNA (Houben et al., 2002). Painting of sex chromosomes has been performed in *Rumex acetosa* by Shibata et al. (1999) and in *Silene latifolia* by Hobza et al. (2004). Hobza et al. (2004) used a modified FAST-FISH protocol based on a short hybridization time combined with a low concentration of probe and succesfully distinguished the sex chromosomes by differential labelling patterns.

Here, by applying microdissection and painting the W chromosome, we found a different labelling region, meaning a different sequence structure, there on the sex chromosome.

MATERIALS AND METHODS

Plant materials and chromosome preparation

Root tips and tender buds of 15 male and 12 female ginkgo plants were used in this study. Slides were prepared by cell wall degradation hypotonic method according to Chen et al. (1979) with minor modifications. In brief, root tips were removed and immersed successively in saturated

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para-dichlorobenzene for 3 h, rinsed in double-distilled water for 30 min, treated with 2.5% (w/v) cellulose and pecitinase (Sigma, Germany) for 30 min, rinsed in double-distilled water for 15 min, and finally fixed in 70% ethanol for 5 min. The fixed material was put on the sterile coverslip (22×60-mm) with a drop of 70% ethanol, using a microscope slide as a carrier to stabilize the coverslip. The material was torn into fine pieces and the debris was removed. Two drops of 70% ethanol were added on the slide and then dried with hot air. Metaphase spreads for FISH were prepared on a common microscope slide.

Microdissection and LA-PCR amplification

Each air-dried chromosome specimen was immediately used for microdissection. The target chromosome, the W chromosome, was isolated using an traditional light microscope (BH-2, Olympus, Japan) equipped with a micromanipulator (MMO-203, Narishige, Japan) and transferred by a fine glass needle pulled by an OC-10 puller (Narishige, Japan) into a 0.5 ml tube according to Li et al. (1998).

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 2 h. The proteinase K was then inactivated at 65°C for 20 min. The chromosomal DNA was digested by *Sau*3A (0.002U in 1×T4 ligase buffer, Takara, Japan) at 37°C for 2 h. The *Sau*3A was inactivated at 65°C for 20 min, and 20 µM 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×Taq buffer, 6 µl of 25 mM $MgCl_2$, 2 µl of 10 mM 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 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 ginkgo genomic DNA was isolated from leaf tissue using the 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 *Sau*3A digested genomic DNA of female and male plants, and the second rounds PCR products from chromosome and controls were transferred onto nylon membranes (Pall, American) after 0.8% agarose gel electrophosis and hybridization with DIG-labeled ginkgo genomic DNA at 42°C for 16 h. The membranes were washed with 0.2×SSC containing 0.1% SDS at 65°C for 30 min. Labeling and detection were performed following the instruction of the Roche DIG High Prime DNA Labeling and Detection Starter Kit II (Roche, Germany).

Fluorescence in situ hybridization (FISH)

FISH was carried out as described by Qi et al. (2002) with minor modifications. The second round PCR products were labeled with DIG-dUTP (Roche, Germany) by randomly-primed DNA synthesis. Hybridization buffer contained 50% deionized formamide; 2×SSC; 50 mM sodium phosphate, pH 7.0; 5% dextran sulfate; 10 ng μl^{-1} probe and 200 ng μl^{-1} unlabelled total genomic DNA. The probe and the unlabelled total genomic DNA were mixed and denatured at 94°C for 10 min before being used. Slides with metaphase spreads were treated with 70% deionised formamide in 2×SSC at 70°C for 2 min. Denatured hybridization buffer (10 µl) was then applied to the slides, which were incubated at 37°C for 2 h. Finally, the slides were washed with 30% deionised formamide in 2×SSC at 37°C for 5 min and twice in 2×SSC at 37°C for 5 min. Metaphase spreads were counterstained with 100 ng ml^{-1} 4’, 6-diamidino-2-phenylindole (DAPI). Fluorescence signal was detected using anti-DIG-fluorescent-conjugate (Roche). The hybridization signals were visualized and recorded using Nikon 80i fluorescent microscope and a cooled CCD camera and were then processed using Adobe Photoshop.

RESULTS

Individual W chromosome microdissection

Using root tips and tender buds as source material and cell wall degradation hypotonic method, we succeeded in preparing good-quality slides of chromosomes in ginkgo. As shown in Figure 1 A, chromosomes were spread evenly on the slide with a low background. The somatic chromosome number was 2n=2x=24, consisting of four metacentrics and twenty subtelocentrics. Chromosome 1, which harbors a satellite, was the biggest chromosome. In male, the satellites of chromosome 1 are homoeomorphic, while in female they are heteromorphic, and one is apparently bigger than the other (Figure 1 A). The chromosome 1 with the bigger satellite was microdissected from the metaphase spreads of female ginkgo root-tip cells with fine glass needle controlled by a micromanipulator (Figure 1) and then used for two rounds of LA-PCR amplification. The southern blot analyses of the second round PCR products confirmed that the products were amplified from the ginkgo genome (Figure 2).

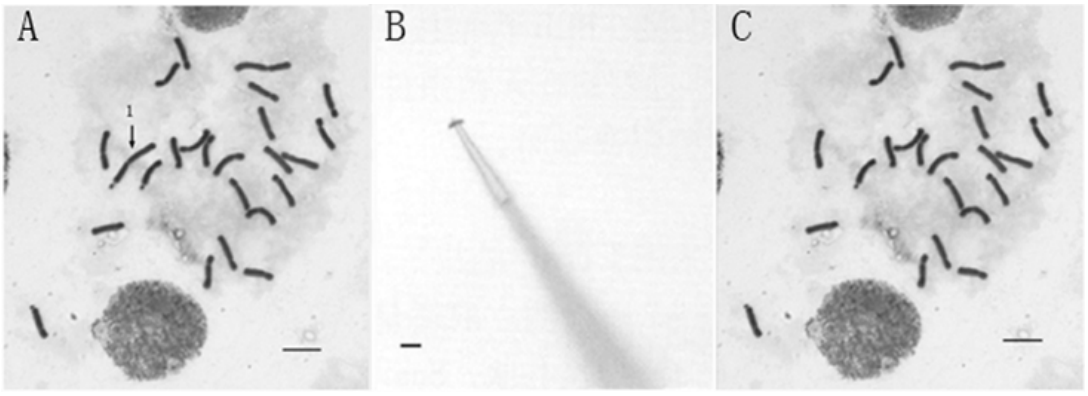

Figure 1. Isolation of an individual chromosome 1 in ginkgo by micromanipulator. A: Mitotic metaphase spread of ginkgo before microdissection. A suitable chromosome 1 with big satellite was found under the inverted microscope (arrow); B: The target chromosome adhering to the tip of a glass needle; C: The target chromosome was removed from the slide. Bar=5, 10, 5 μm, respectively.

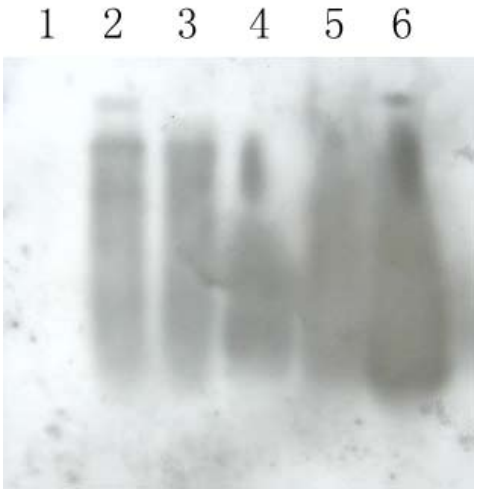

Figure 2. Southern blot hybridization analysis of second-round LA-PCR products with DIG-labeled genomic ginkgo DNA. Lane 1 was the negative control (no DNA template in LA-PCR), lane 2-3 were the *Sau*3A digested total genomic ginkgo DNA of female and male respectively, lane 4-5 were the LA-PCR products, and lane 6 was the positive control (genomic DNA as template in LA-PCR).

Chromosome painting of W chromosome

Blocking with twenty-fold excess of unlabelled genomic DNA, the DIG-labeled second-round LA-PCR products originating from individual microdissected chromosome 1 were hybridized to mitotic metaphase spreads. Signals were mainly observed along the entire W chromosome while along about 1/2 length at the end of the long arm of the Z chromosome, the signals were weaker than at other parts of the Z chromosome. At the same time, some signals were also observed on the terminal, centromeric, or other regions of other chromosomes (Figure 3).

DISCUSSION

In plants, 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. Several experiments with DOP-PCR amplified probes from microdissected chromosomes or chromosome regions hybridized to metaphase chromosome complements, unequivocally revealed dispersed hybridization signals

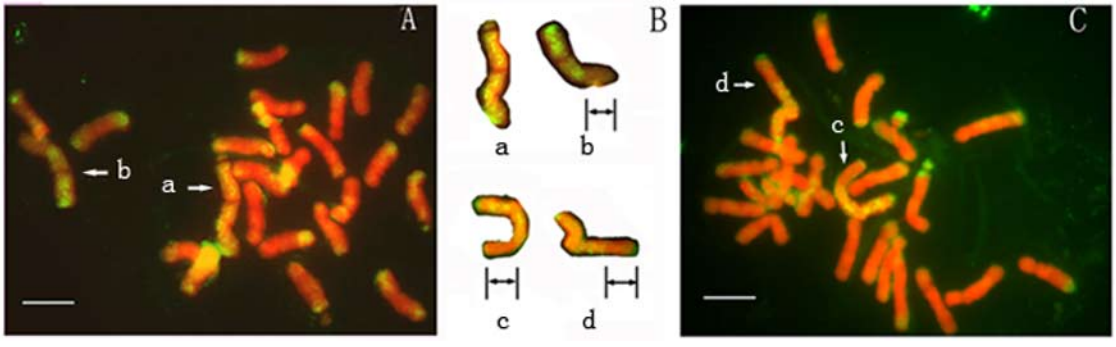

Figure 3. Chromosome painting patterns on female (A) and male (C) metaphase chromosomes of ginkgo using DIG-labeled second-round LA-PCR products. chromosome marked as "a" was chromosome 1 with big satellite while "b, c, d" were chromosome 1 with small satellite. FISH signals on the segments marked with arrows in B were apparently weaker than other segments of chromosome 1. Bar=5 μm.

on all chromosomes in the case of *Vicia faba, Hordeum vulgare, Triticum aestivum, Picea abies* and *Petunia hybrida* (Fuchs et al., 1996). In our study, we modified the FISH procedure including changing the hybridization time and the amount of DNA probe or using unlabelled genomic DNA for blocking (data not shown). The results were still similar to the cases described above that all chromosomes harbor signals. However a clear difference emerged between the intensity of the signal on the chromosome of probe origin and that of the other chromosomes. Such uniform hybridization patterns were found irrespective of the blocking conditions and the concentrations of genomic DNA used in these experiments. The number of repetitive sequences in plants is too large for efficient blocking by conventional prehybridization procedures. In the nuclear genome of higher plants, especially in the repetitive sequence, most cytosine are methylated. The unmethylated loci maily disperse in the low and single copy sequence regions. Methylation sensitive restriction enzyme cannot recognize the methylated cytosine loci. If it could, then low and single copy sequences would be relatively rich in the products. Many researchers used the methylation sensitive restriction enzyme such as S*au*3A to digeste the DNA. The LA-PCR method also can concentrate the low copy sequences, and it can amplify longer fragments than DOP-PCR.

In plants, chromosome painting is possible only on specialized chromosomes, such as the B and Y chromosome or on chromosomal regions that contain specific highly repetitive sequences. Painting of sex chromosomes has been successfully performed in *Rumex acetosa* by Shibata et al. (1999) and in *Silene latifolia* by Hobza et al. (2004). In *Rumex acetosa*, strong signals were abserved on the Y_1 andY_2 chromosomes, and weak signals were also observed on the X chromsome and autosomes. In *Silen latifolia*, Hobza et al. (2004) found that the X chromosome probe revealed a clear signal on the entire X chromosomes while the signal on the other chromosomes, including the Y, was of lower intensity. Similar results were obtained with the Y chromosome probe when the Y chromosome was strongly labeled. The different labeling patterns of sex chromosomes showed that the composition of the DNA sequences in the X and Y chromosome differs. In our study, different labelling patterns have been shown on the sex chromosomes of *Ginkgo biloba*. Along about 1/2 length at the end of the long arm of the Z chromosome, the signals were weaker than at other parts of chromosome 1. This indicated that the composition of DNA sequences in the W chromosome and the Z chromosome might differ, especially in the parts where the FISH signal intensity differed. We believed this part on W chromosome became specialized as a result of the accumulation of chromosome-specific repetitive sequences in the process of sex chromosome evolution. It would harbor sex-specific sequences and might be a non-recombining region. Further study, such as construction of a W chromosome-specific genomic library and isolation of female specific DNA markers would reveal more information about the evolution and divergence of the sex chromosomes in *Ginkgo biloba*.

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銀杏 W 染色體顯微分離及螢光原位雜交分析顯示性染色體不同雜交帶型

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以雌性銀杏（*Ginkgo biloba*）根尖細胞為材料，採用玻璃針分離法，通過顯微操作器成功地分離出銀杏 W 染色體。將分離得到的 W 染色體去蛋白、*Sau*3A 酶切，進行 LA-PCR 擴增。以 DIG-dUTP 標記的銀杏基因組 DNA 為探針進行 Southern 雜交，結果表明顯微分離出的染色體擴增片段與銀杏基因組 DNA 同源，從而證明 W 染色體 DNA 確實已被成功擴增。以 W 染色體第 2 輪 PCR 產物為探針，在適當條件下對銀杏雌雄中期細胞進行螢光原位雜交，發現螢光信號較密集分佈於 W 染色體，Z 染色體長臂末端 1/2 的區域的信號強度則比其他部位弱，同時螢光信號也出現在其他染色體的著絲粒及端粒等區域。該結果表明，銀杏的 W 和 Z 染色體的序列結果存在差異，特別是在長臂末端 1/2 區域處。這個區域可能是一個雌性特異區域，是在性染色體進化過程中逐漸形成的。

關鍵詞：顯微分離；染色體繪圖；螢光原位雜交；銀杏；性染色體。