IDENTIFICATION OF RAPD MAKERS LINKED TO THE MALE SEX IN DIOECIOUS *GINKGO BILOBA* L.

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Abstract：The random amplified polymorphic DNA (RAPD) technique was used to amplify DNA fragment ,aiming at finding markers linked to the sex determination in *Ginkgo biloba* L. Of the 158 RAPD bands , only a 1080 bp RAPD marker generated by aprimer (S2) of random sequence ,which was found to be associated with the male plants. which was abundant in AT and also used to design flanking 24-mer oligonucleotide SCAR primers SCS2-1 and SCS2-2. Both of these SCARs amplified a single 1080bp fragment; the same size as the cloned RAPD fragment. This SCAR marker proved to be suitable for precise and rapid identification of male plants in the early stages for breeding programs.

INTRODUCTION

Ginkgo biloba , a dioecious perennial gymnosperm ,is the only living representative of the order Ginkgoales left in the Tertiary (65 million years ago) (Major ,1967) . It is therefore called the“living fossil”. In *Ginkgo* , male and female trees have different values in practical application. Usually male trees are used for street and landscape trees , female plants are not desirable for this purpose , because their seeds produce unpleasant odor and some poisonous substances when fallen and decayed. On the other hand , *Ginkgo* seeds are supposed to cure asthma , coughs , bladder irritability , blenorrhoa and uterine fluxes (Jacobs and Browner , 2000) . The time when young Ginkgo trees begin to flower intervenes between 15 to 25 years of growth in nature. In addition , it is not very easy to distinguish the fertile spur shoot from the common vegetative spur shoot before reproductive time (Fu and Yang , 1993 ; Hara , 1997) . Therefore , sex identification in the early stages of plant life is of great importance in the strategy of plant management and utilization.

The debate on sex chromosomes in Ginkgo has gone on for about half a century. Lee (1954) and Pollock(1957) ascribed that Ginkgo possessed an XY type of sex determining mechanism. But Chen et al (1993) attributed the WZ type to the sex determining mechanism. Therefore,the type of sex determining in Ginkgo is still a suspensive question.In addition, Botanists and biochemists have studied the sex of Ginkgo in several ways(Yadav et al ,1985;Zhong et al ,1982;Du et al ,1997).However, there is no affirmed conclusion until today.

Genetic marker systems based on direct analysis of the genomic DNA have not only been useful in breeding programs but also have allowed to understand the genetics of dioecism in many dioecious plants (Ainsworth ,2000) . The RAPD technique has been used to amplify DNA fragment in order to find out the possible markers linked to the sex determination in several dioecious plants , such as *Silene latifolia* (Mulcahy et al ,1992 ; Zhang et al , 1998) , *Cannabis sativa* (Ottó Törjék et al.2002), *Humulus lupulus* (Polley et al. 1997), *Carica papaya* (Parasnis et al. 2000;Urasaki et al.2002) ,*Salix viminalis* (Alstrom-Rapaport et al.1998; Gunter et al.2003) , *Rumex acetosa* (Helena Korpelainen et al.2002) and *Mercurialis annua* (Khadka et al.2002). The objective of this study is to identify molecular markers linked to sex determination in Ginkgo using RAPD. We here report the findings of a RAPD marker linked to male plants that is not present in female plants.The importance of the findings in the early identification of sex in Ginkgo as well as the possible implications in understanding the molecular basis of sex determination in Ginkgo are also discussed.

MATERIALS AND METHODS

Plant materials

Ginkgo biloba L. was grown under natural light and photoperiod in an experimental field at the Henan Normal University. After determining the sex types of the plants , individual leaf samples were collected (about 1 g) and stored at –80°C.

Genomic DNA extraction

Genomic DNA was extracted separately from each samples using the cetyltriethyl Ammonium bromide (CTAB) method (Doyle and Doyle.1987) with minor modifications. About 0. 3 g leaf tissue was ground to a fine powder in liquid nitrogen and mixed with 700μL of CTAB extraction buffer (100 mMTris-HCl (pH8.0),1.4 MNaCl ,20mMEDTA(pH8.0),0.5%$NaHSO_3$,2%CTAB,1%β-mercaptoe- thanol,1%PVP)in a 1.5 mL Eppendorf tube. The mixture was incubated at 65°C for 1 h , then an equal volume of chloroform-isoamylalcohol (24μ1) was added, and centrifuged at 10000 rpm for 10 min. The aqueous phase was repeatedly decanted and transferred to a fresh tube to reduce impurity between two phases. Extraction steps were repeated using phenol-chloroform-isoamyl alcohol(25:24:1) mixture

and then chloroform-isoamylalcohol (24μ1) . The last aqueous phase was mixed with 2/3 of isopropanol and 1/10 of 2.0 M NaAc (pH5.2) at -20°C for at least 1h to precipitate DNA and centrifuged at 8000 rpm for 15 min at 4°C. The nucleic acid precipitate was washed with 70% ethanol , air-dried and suspended in 200μL of ddH_2O containing 10mg/mL RNase A at 37°C for 1h. The extracted DNA was diluted in ddH_2O to 40ng/μL and subjected to polymerase chain reaction (PCR) amplification.

An equal amount of DNA from 10 male and 10 female plants was combined to form DNA pools.

RAPD reaction

PCR amplification reaction was performed in 25μL containing 40ng genomic DNA,10mmol/L Tris-HCl (pH9.0 at 25),50 mmol/L KCl,2.0 mmol/L $MgCl_2$,200μmol/LdNTP,0.5μmol primer and 1.5 unit of *Taq* DNA polymerase. DNA amplification was performed in Gene Amp PCR System 2400 thermal cycle (Perkin-Elmer Corporation,USA) , with the following cycling parameters :45 cycles of 1min at 94°C, 1 min at 36°Cand 2 min at 72°C, followed by one cycle of 5min at 72°C and then hold at 4°C prior to analysis. Amplification products were analyzed by gel electrophoresis in 1.5% agarose (BBI,Canada) in 1×TAE buffer. Gels were stained with ethidium bromide and visualized on a UV transilluminator. Each amplification reaction was performed using a single primer and repeated at least three times in order to verify the reproducibility of the results.

Fragment isolation, cloning, sequence analysis and SCAR primer design

The putatively female-associated band amplified by RAPD analysis was excised from the gel using UNIQ kit (Sangon,shanghai,China), cloned into the pGEM-T vector (Promega,USA), and the plasmids then was transformed into *Escherichia coli* JM109 cells (Dingguo Ltd. , Beijing , China) . The programme is referred to Molecular cloning (Sambrook 1989).The same DNA fragments in the cloned form were sequenced by dideoxynucleotide chain termination method using T7 and SP6 as forward and reverse primers respectively. The DNA sequence was analyzed using the BLAST program (NCBI,National Center for Biotechnology Information,USA). The ends of the cloned fragment sequences were used to design 24-mer primers for amplification of the fragment from genomic DNA. The 24-mer primer sequences are listed in *Table 1*。

Table 1. SCAR markers constructed from one sex-linked sequences in *Ginkgo biloba* L

RAPD primer	SCAR marker	Sequence 5'–3'	annealing temprature
S2	SCS2-1	TGATCCCTGG CAAGGTCACC AAGT	65℃
	SCS2-2	TGATCCCTGG CCACTACAAC GACA	

RESULTS AND DISCUSSION

RAPD pattern

To identify DNA markers linked to the sex locus, 100 RAPD primers were screened. The primers produced 550 bands with each primer generating 3 to 8 bands, and 5.5 bands on average. The banding patterns of most primers showed no difference between the pooled male and female samples , two primers produced at least one fragment that was different between the two pools. When amplification reactions were carried out with DNA from the individual, one band amplified by primer S2 (5'-_TGATCCCTGG -3') appeared to be sex-associated. It was present in male while absent in female plants (Fig. 2). The other primer produced the band patterns that could not be consistently repeated. In order to determine if the marker could be broadly applied as indicators of sex, both were then tested in an additional 30 male and 30 female plants. the band was generated in all the tested 30 male individuals while absent in all 30 females.

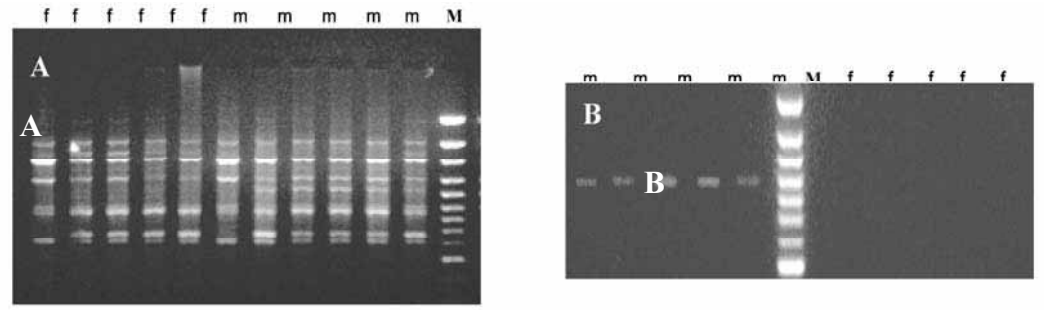

Fig.1. A , male-specific DNA fragments produced by PCR with S2 primers in different plants. (Arrows indicate sex linked fragments.) .**B** , **SCAR** gels. (The lanes marked ‘m’ and ‘f’ represent DNA amplified from individual male and female plants. Lanes marked ‘M’ contains molecular size markers SM0321 , MBI)

DNA sequence analysis

The band was cloned and the sequence was found to be 1080 bp in length , and the G + C content is 39.70%(Genbank number AY971577) (fig.2). A search of databases ,GenBank,EMBL, DDBJ, PDB (but not EST,STS,GSS,or phase 0,1 or 2 HTGS sequences) showed that the sequence of the band was low in homology to any known sequences . The sequence did not contain a recognizable open reading frame. The BLAST search suggested that the band was probably in a non-coding region of the genome.

TGATCCCTGG CAAGGTCACC AAGTGCAAGA GGGGATTTTC TCTTTTTTTT
CTTTTTCTTG AAGTACTAAG TGATGTATTA GATTTTGGGT GGGAAGTTAG
ATGTGGGGCC TTGACACATT CAATGTCATC ACTTTTGCAA GGAGGTGATT
CTTGAGAAGT TTAGGATGAT ACCAAAGGCA TCAAGAAGAC AAGAATAACA
CTTAAGGAGG AGGTCGAAGA GATATTACAA ACCAAATAAT CTAGGTCATA
AGTGGGGACT AGACTATCAT GTGGCATCCC CATTCTAGGA GGATTTTTTA
AGGAAGGACC TTTGTGTTCG TTCCTGCTTC AAGCTCTTAT CTTGAGATCC
TAGGAGATTA TATTCTTGCT TGTTTAGAGG GAAAGCTCAA GCATGCTCTC
ATTCCTTATG GGAGTTACTA ATGTGTCCAC TTCCTTCACT ATCCTCTACA
CCTCTACTTC TATAATGACT ATGAACACTT ATTCCTAGAT TTTCAATGGG
ATATCGCTTT GAAACTCTGA CATTTATGTT GTTGTAACCT GCTATAAATA
CCGTCCAAGT TAGACCCCCT TACTTTATTT TTTTTTGTTG GCCAAACATC
TTAGGGGGTG AAAGAACTTT TGGAAGGACT AGGAGGGGTA TATATTCTAA
TATACATTTC CTCCTTAGTA CCAAATTCCT TCTTGGTTTG TCACAACTCT
CCTTGGTGTG CCAAGACTTC CCAAACTCAC CAAGAGGTGT CTTGTCTATT

CATCTTTTAG AACACATGCC TTAAGAATTT TTAAGTGACT ATGTGGTTCC
CTTACTTAAT TAGTATTCTC TTGGTCTCCA TTTACCTTCT CAAATATCCT
ATGAATGCTT CTATCTCTCT AAGATATAGA TATTTCCTTG CCATGCATAT
CCTGGGTGAT ACTAGCCTCT TTTGATTATA CTAGGACTGT ATCCAACTCC
TAGGAGATTG TTGACACTGG AAAACCCCAC CGCGCGCCCA CCGACACGCG
ACGGTAGAAA TCCGCGTCAA CAACCTCGAT TTTTAAATTT TTAATTGAAT
TATCGT**TGTC GTTGTAGTGG CCAGGGATCA**

Fig.2 Sequence of the cloned S2 fragment
(The underlined sequences represent the site of designed SCAR primers.)

DISCUSSION

In this study we got the male-associated marker from the pooled DNA in the campus of Henan Normal University ,which was present only in males and absent in females.This marker was also stable in male plants in luoyang and dengfeng . This suggested that it was linked to the sex determination in Ginkgo and was not affected by the environmental conditions. The research suggested that S1478-682 could be considered to be a reliable genetic marker in sex determination in Ginkgo and could be used to satisfactorily detect the sexuality of Ginkgo.

Ginkgo has a large haploid genome of approximately 19. 5 $\times 10^9$ bp (Murry , 1998) and the genetic mechanism of sex determination is still unknown. It was no wonder that only one male-associated band was found after screening 100 random primers. The low level of DNA polymorphism detected in this study , together with the large number of common bands between the male and female plants , implies that the genomes of the two sexes are rather homologous. Jiang et al (2003) got 2 male-specific markers screening 1200 primers.Wang et al (2002) compared the genomes between two sexes of Ginkgo and considered the homology between two sexes 99. 98 %. The low frequency of sex-linked bands might indicate that the DNA segments involved in sex determination are very small , and probably represents a single gene , or very few genes (Hormara , 1994) .

These sequences are probably from a non-coding genome region, as they contain no large open reading frames. Based on the present data, however, it is impossible to ascertain if the sequence is part of the gene(s) for sex determination in *Ginkgo biloba* L. Although the RAPD marker was amplified only from male plants, partial sequence of this band might be common to both male and female genomes. Southern blot (data not shown) using the amplified or cloned fragment as a probe against genomic DNA from both male and female plants produced a high background signal in both sex samples. This might be explained by supposing that the differences at the DNA level are limited in the region including the sequence of the RAPD primer originally used. Such hybridization results are consistent with the presence of repetitive DNA sequences. These results are consistent with those of previously studied male-specific DNA fragments in plants (Ruas,1998;Gill,1998).

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