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RESEARCH ARTICLE

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RAPD markers related to sex locus in *Populus tomentosa*

Abstract By using the methods of random amplified polymorphic DNA (RAPD) and bulked segregate analysis (BSA), we identified markers that are linked to the sex determination in the dioecious *Populus tomentosa*. Male and female bulks were created through rough mixing equal amounts of its five individual DNA. A total of 88 primers were screened. Twelve primers produced clear patterns with at least one band that appeared to be polymorphic between the two bulks. Subsequently, five male and female individuals were analyzed with those 12 primers, and only S60 (ACCCGGTCAC) could generate a common 1800 bp DNA fragment in all five male individuals and male pool but not in any female individuals. It can be concluded that the gender of *P. tomentosa* is most likely connected to the S60-1800 bp DNA fragment and RAPD markers. S60, therefore, can be used for selecting the gender of *P. tomentosa*.

Keywords biotechnology, RAPD, bulked segregate analysis (BSA), *Populus tomentosa*

1 Introduction

Populus tomentosa, a major species for afforestation in the Yellow River Basin, is important in the urban landscape. It has become the preferred tree in the landscape for the unique characteristics of its branches, stem and leaves, and constituted a high proportion of the road and garden greening design. *P. tomentosa* is dioecious, and the male and female plants have different economic value. The male is higher than the female in growth potential, so it is applicable as a shelterbelt forest species. The female's flying-butt pollution leads to many environmental problems, so we should select the gender of *P. tomentosa* for cultivation in the breeding. However, it is difficult to identify the gender by external morphological traits, for the gender is only apparent during the period of flowering and fruiting.

Previous researchers mostly studied the gender identification on the differences of the mature individuals in morphology, peroxidase isozyme, physiology and biochemistry, amino acid contents and nuclear analysis (Dong et al., 2007). In addition, the gender differentiation of dioecious plants is a complex system. The sex identification based on gene expression products is inaccurate and unreliable, and cannot be primarily applied to the early gender identification of dioecious plants (Yin et al., 2003). Recently, DNA molecular markers, which are not affected by the development stages, environment, specific tissues and can be used for multi-locus association study, are widely used in early gender identification. It has been successfully applied to the gender identification of *Cannabis satival* (Mandolino et al., 1999; Chen et al., 2001), *Ginkgo biloba* (Wang et al., 2001; Wang et al., 2002), *Carica papaya* (Parasnis et al., 1999), *Actinidia chinensis* (Gill et al., 1998) and other plants.

Michelmore et al. (1991) first proposed the BSA (bulk segregant analysis) in 1991, and had identified three RAPD markers linked to the downy mildew resistance gene of *Lactuca sativa*. Compared to the general approach, we can quickly and effectively identify the molecular markers linked to the genes of particular traits by BSA without constructing the near-isogenic lines (near-isogenic line, NIL). As some certain regions of the genome were not isolated for a single group, it is difficult to increase the number of makers and the mapping density. However, we can identify some specific genes located in the sparse region of markers. At present, this method has been applied to the research of resistant gene molecular markers of many crops, such as *Triticum aestivum* (Suo et al., 2001), *Pisum sativum* (Mahboob et al., 2001), *Solanum lycopersicum* (Chague et al., 1997) and so on, but there were very few studies on trees. In 1996, Grattapaqlia et al.

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(1996) proposed that he had identified the RAPD makers related to the tree volume growth by using bulked segregate analysis (BSA). In 1999, Cristofani et al. (1999) reported two RAPD makers linked to a locus involved in the root rot resistance genes of *Citrus reticulata*, and the genetic distances were 17.8 and 23.1 cm.

P. tomentosa, which is a dioecious plant of white poplar in Salicaceae, has an important economic and greening value, but there are difficulties in early gender identification. In this study, by using the methods of RAPD and bulked segregate analysis, we tested the polymorphism between male and female trees. We tried to search for stable sex-associated markers of *P. tomentosa* for the early gender identification, and provide some molecular foundation for the cultivation and urban greening.

2 Materials and methods

2.1 Plant materials

Male and female individuals of *P. tomentosa*, were usually used for hybrids in the laboratory of forest genetics and breeding in the School of Forestry, Northwest Agriculture and Forestry University. The first young leaf in fresh and healthy spurs was picked, marked with the gender and stored in an ice box for DNA isolation.

2.2 Methods

2.2.1 DNA extraction

Total genomic DNA was isolated from 0.5 g of leaf tissues of female and male individuals separately with the modified CTAB method (Dellaporta et al., 1983). The leaf was scrubbed with 70% alcohol, removed of vein, ground rapidly with liquid nitrogen and put into a 5-mL centrifuge tube. Then, the mixture was added by 1950 μL CTAB extraction buffer (2% soluble PVP, 2.5% *β*-2-mercaptoethanol), gently mixed, and insulated in water bath at 65°C for 40 min (gently shaken every 5 min). After that, it was cooled to room temperature rapidly, and was co-mixed with an equal volume of the mixture (V_{phenol}: $V_{\text{chloroform}}$: $V_{\text{isoamyl alcohol}}$= 25:24:1), stabilized for 5 min, centrifuged at 2100 r/min, 40°C for 30 min. Four hundred μL of the supernatant and pre-cooling 2/3 ethanol were added into 1.5-mL centrifuge tubes and gently shaken, and then stabilized at −20°C for 1 h, centrifuged at 12000 r/min for 5 min. The supernatant was discarded. The pellet was washed with 75% ethanol three times and the centrifuge tube was insulated in water bath at 56°C for 10 min, so that the liquid completely evaporated. We added 100 μL TE to the tube, resuspended the sediment, insulated it in water bath at 56°C for 10 min, shocked blending, centrifuged at 12000 r/min for 2 min, and prepared the supernatant for use.

2.2.2 RAPD reaction and electrophoresis analysis

Referenced to the methods of Williams et al. (1990), the PCR reactions were carried out in a 20-μL volume containing one unit of Taq polymerase (MBI), 50 ng of genomic DNA, 0.1 mmol/L each dNTPs, 2.5 μL of 10 × PCR reaction buffer [500 mmol/L KCl, 200 mmol/L Tris-HCl (pH 8.3)] and 2 μL Mg^{2+} (25 mmol/L), 1.6 μL dNTP (2.5 mmol/L each dNTPs), 1 μL of RAPD primer (10 mmol/L) and double distilled water, DNA amplifications were carried out in a PTC 2200 controller. The following steps were used for RAPD: a predegeneration of 2 min at 94°C at followed by 38 cycles of 30 s at 94°C, 15 s at 36°C, and 90 s at 72°C, and a final cycle of 7 min at 72°C. The amplification products were resolved on 1.2% agarose gels in 1× TAE buffer and stained with 0.5 μg/mL ethidium, and the results were analyzed by gel imaging system.

2.2.3 DNA pools

To test the genomic DNA polymorphism between male and female, five male individuals and five female individuals were selected randomly to make a male DNA pool and a female DNA pool, respectively, for RAPD primer screening. The RAPD primers, which revealed the DNA polymorphism between the male and female pools, were selected to be used for individual analysis of randomly selected male and female plants.

3 Results and analysis

3.1 Polymorphism of RAPD primers between male and female bulks

By using the methods of bulked segregate analysis (BSA), we divided the materials into two pools. A total of 88 primers were used for RAPD analysis of male and female individuals, and 12 primers gave some differences between male and females (Table 1, Fig. 1).

Table 1 Random primers producing polymorphic bands between male and female bulks

number	sequence (5′–3′)	number	sequence (5′–3′)
S21	CAGGCCCTTC	S60	ACCCGGTCAC
S22	TGCCGAGCTG	S303	TGGCGCAGTG
S24	AATCGGGCTG	S304	CCGCTACCGA
S38	AGGTGACCGT	S306	ACGCCAGAGG
S45	TGAGCGGACA	S426	GAGACGCACA
S48	GTGTGCCCCA	S429	TGCCGGCTTG

3.2 Sex-associated RAPD makers

In order to get stable sex-associated RAPD makers, five male individuals and five female sex-specific individuals

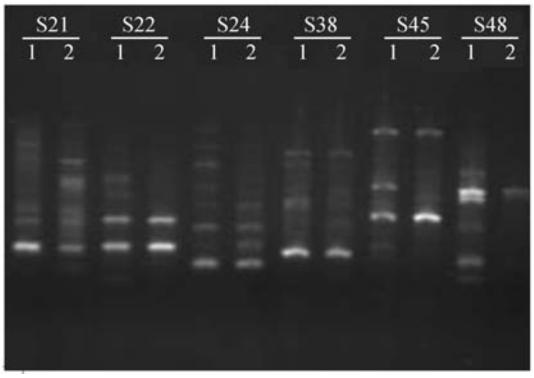

Fig. 1 Polymorphism of RAPD primers between male and female bulks. Lane 1: amplification result of the female bulk; Lane 2: amplification result of the male bulk.

were selected for RAPD analysis of the 12 primers, which shows polymorphism between the male and female pools. The unique bands of 10 primers male and female bulks were a bit confusing. The primer S303 performed clearly in the male and also performed in the female, while the primer S426 performed clearly in the female and also performed in the male. Only one primer, S60 (ACCCGGTCAC), was found to have sex specificity in bulk analysis. S60 produced a unique 800 base pairs fragment in the male bulk, but this band was absent in the female bulk (Fig. 2).

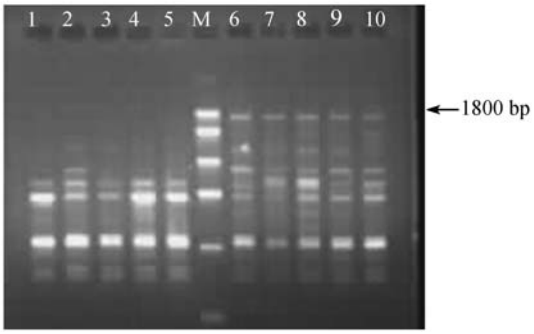

Fig. 2 Amplification result of individual male and female samples with the primer S60. Lane 1: amplification result of the female bulk; Lane 2: amplification result of the male bulk.

4 Discussion

4.1 The primers

Fritsch et al. (1993) found that the amplification strength of primers was related to their own sequences, but independent with the source of DNA. The coefficient correlation between the same primer and different DNA sources for analysis is 0.694, that is, one primer can produce strongly amplified products in one DNA sample, its applicable probability for another DNA sources samples is close to 70%, which has important reference value on the choice of primers. All the primers used in this study can produce unique bands, which provides basis for effective choice of primers.

4.2 Plant gender

Sex determination of the plant is a complex process and influenced by many factors. Generally, plant's sex-determination mechanisms in the following: 1) determination by sex chromosome, 2) determination by the ratio of autosomal and sex chromosomes, and 3) regulation by hormone. As a result, genetic differences may exist between the male and female trees of *P. tomentosa*, which provided the basis for gender study with RAPD technology. Genome DNA of male and female plants are equivalently mixed to make a male DNA pool and a female DNA pool for the RAPD analysis between genders, which can decrease the occurrence of false positives (Miklas et al., 1993), as well as rapidly detect the genome difference between the male and female.

4.3 The necessity and methods for conversion of RAPD makers

Random amplified polymorphic DNA (RAPD) technique, which is based on the PCR technique, is a simple, rapid and effective technique of DNA molecular markers used in detecting DNA mutation on genome level with high sensitivity, but lack of stability. In recent years, there are a lot of researches of identification on such method (Zhang et al., 2001; Hu et al., 2002; Li and Yang, 2003; Zhang et al., 2004; Chen et al., 2005; Huang et al., 2005; Yang et al., 2005). The primers of RAPD are usually 10 bp random primers, with low capacity of combining with the specific target sequence, and the probability of the non-specific amplification band is higher. In addition, most RAPD markers are dominant and unavailable in distinguishing between dominant heterozygote and heterozyote. Therefore, it is necessary to convert RAPD makers. The conversion methods are as follow: 1) use the specific amplified sequence in PCR procedural as a probe for hybridization, transform it into RFLP markers; 2) PCR product will be cloned, sequenced, we designed 20 to 24 base pairs of specific primers according to the two ends of the sequence, which has translated into a total of dominant effect of the STS markers (sequence-tagged site) or SCAR markers (sequence characterized amplified region). SCAR marker is a single-nucleotide marker, with low sensitivity to the reaction conditions, amplification is a product of a single specific amplification. The DNA can be amplified to determine the identification of genotype. This test will use the RAPD markers and the more specific SCAR markers for the further study of gender of *P. tomentosa* to be used to provide more reliable molecular markers.

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