

Identification of Rapd Markers Linked to Thickness Gene of Shuck in Walnut

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Abstract: The aim of this study was to develop thickness molecular markers for thin and thick walnut to detect their proper identification. RAPD marker T16₁₂₂₄ was generated using a PCR-based RAPD technique by Bulk Segregant Analysis (BSA), which was probably related to the precious characteristic for thickness of walnut. Sequence-characterized Amplified Region (SCAR) marker was developed from T16₁₂₂₄ sequences, using 22-mer oligonucleotide primers designed from the RAPD primer. The SCAR primers amplified the target sequences only in the thin walnut pool, which was used to amplify each individual and the result showed that the pattern of this SCAR marker was the same as that of the RAPD marker.

Key words: Walnut • Bulk Segregant Analysis (BSA) • RAPD marker • thickness character • SCAR marker

INTRODUCTION

Walnut is one of the oldest agricultural tree crops and is cultivated for both nuts and timbers. In recent years, molecular markers have been developed in walnuts. They include randomly amplified polymorphic DNA (RAPD) [1], amplified fragment length polymorphism [2] and sequence characterized amplified regions (SCAR) [3]. The results of this study showed that walnut cultivars have a high degree of diversity and that the markers employed are efficient for detecting polymorphisms in walnuts.

A number of PCR-based methods, including randomly amplified polymorphic DNA [4, 5] and amplified fragment length polymorphism [6], are available that do not require previous sequence information of the genome to be studied. In a RAPD assay, a short, usually ten nucleotides long, arbitrary primer is used, which generally anneals with multiple sites in different regions of the genome and amplifies several genetic loci simultaneously. This technique is simple, relatively inexpensive and has been employed to analyze the intra and intergenetic genetic diversity of plants. In contrast to RAPD, the AFLP technique generates relatively complex patterns.

To overcome the reproducibility problem associated with the RAPD technique, RAPD markers have been converted into sequence-characterized amplified regions [7]. SCAR markers have been developed for several crops, including lettuce [7], common bean [8], Raspberry [9], grape [10], rice [11], *Brassica* [12] and wheat [13]. Although molecular genotyping through SCAR primers

has been documented in a few woody species, e.g. olive [14], papaya [15], apple [16] and *salix* [17], we still have many difficulties to deal with in woody study.

Like other woody perennial plants, walnut seedling has a long juvenile phase before starting to bear nuts. Therefore breeders have difficulties in improving early selection efficiency in the program of cross breeding. DNA marker is a promising way to assist selection (MAS). Since seedlings be reselected for traits of importance before the trees mature. This may save much cost and time for field maintenance of the plants and evaluation of the traits of interest.

As a rapid, simple and low cost technique, RAPD has been used for screening DNA markers of some important agronomic traits in fruits. So, in this study, RAPD marker tightly linked to thickness gene would be selected using BSA Method, then sequences the marker fragment and convert it into a stable SCAR marker. Research and employment of this has important meaning not only for the varieties improvement of walnuts, controlling and realizing of thickness quality to provide a tool for high accuracy marker assistant selection in walnut breeding, at the same time, to set a good background for map based cloning of the thickness gene.

MATERIALS AND METHODS

Young leaf samples were collected as the following standards: thin walnut is a tree with nutshell less than 0.10cm and complete, while thick walnut is a tree with nutshell more than 0.20cm. Both the thin and thick

samples were collected from Liangshan Autonomy Continent in Sichuan, which were dried with silica gels.

DNA extraction method suitable for RAPD analysis has been established by improving the CTAB method. About 0.2g dry leaves were ground to fine powder with PVP₄₀ (polyvinyl pyrrolidone) in liquid nitrogen and then transferred to the 10ml tube containing 4mL extraction buffer (200 mmol/L Tris-HCl, pH8.0, 50 mmol/L EDTA, 250 mmol/L NaCl) and 120 μ L β -BME (β -mercaptoethanol), 4°C for 10-30min. The mixture was centrifuged at 5000rpm for 10 min, the supernatant was poured off. And extracted the deposit using a high-salt extraction buffer 5mL (100 mmol/L Tris-HC, pH8.0, 1.4 mol/L NaCl, 20 mmol/L EDTA, 2CTAB) and 2 μ L β -BME (β -mercaptoethanol). The homogenate was incubated at 65°C water bath for 45 min. The mixture was centrifuged at 5000rpm for 10 min and then the supernatant was transferred to a new tube and purified with equal volume of chloroform and isoamyl (24:1), mixed well by inversion and shaken gently for 2-3min. Repeat above step twice. After that, the mixture was centrifuged at 5000rpm for 10 min and then the supernatant was transferred to a new tube and twice cold isopropanol was added to precipitate DNA. After 1 0-1 5min at -20°C. And the DNA pellet was washed twice with 70 ethanol. Finally, DNA was dried at room temperature and resolved in 200-400 μ L TE (pH8.0) buffer, conserved at -20°C.

The concentration and quality of each sample of DNA were calculated from the optical density (OD) values at 230, 260 and 280 nm and DNA was regarded as being of good quality when the ratios of OD_{260/280} and OD_{260/230} were near 1.8.

Two different DNA bulks were prepared from equal volumes of standardized DNA (20 ng/ μ L) from five thin walnuts and five thick walnuts respectively based on the thickness data. The 200 primers were used to simultaneously screen between the two different DNA bulks from thin and thick plants and they were tested subsequently in the individuals for determining the presence or absence of coupling-phase markers linked to thickness.

The reaction was carried out in a volume of 25 μ L and was prepared as follows: 20ng of genomic DNA, 1U TaqE, 2.0mmol/L MgCl₂, 2.5 μ L 10 \times reaction buffer, 3.25mmol/L dNTPs and 0.36 μ mol/L primer. Each reaction solution was overlaid with one drop of mineral oil to prevent evaporation. Amplification reactions was performed in a 96-well thermocycler (Eppendorf Authorized Thermal Cycler PCR) programmed as follows: an initial denaturizing at 94°C for 5 min followed by 40 cycles of 45s at 94°C, 45s

at 37°C, 90s at 72°C and finally extended at 72°C for 10min. The amplified products were analyzed for band presence and absence after electrophoretic separation on 2% agarose gels and staining with ethidium bromide. Each amplified reaction was carried out three times to ensure result consistency.

RAPD marker fragment was excised from agarose gel with a sterile gel slicer and the DNA was purified using gel extraction kit. The eluted fragments were ligated into pUCm-T Easy vector following the supplier's instructions, transformed into competent *E.coli* strain cells (DH5 α) and culturing. The plasmid DNA purified from the white colonies. Selected transformed clones were screened by PCR analysis with corresponding RAPD primer. The inserted DNA fragments were sequenced by Invitrogen Biotechnology Co., Ltd.

A pair of SCAR primer was designed and synthesized by Invitrogen Biotechnology Co., Ltd. One forward and one reverse primer were designed according to the result of sequencing by primer. The primers sequences were as follows:

P1: GGTGAACGCTTAAAGAAGCTCC

P2: GGTGAACGCTGCCAAGATATC

SCAR-PCR amplification reaction was conducted using the above mentioned methods except that the annealing temperature was increased to 55°C and cycle number was 28.

RESULTS AND DISCUSSION

Of the 200 RAPD primers screened, 13 primers produced distinct, reproducible, polymorphic profiles between the two bulks surveyed. The approximate size range of the RAPD products was 250bp to 1800bp. Reproducibility of the amplification pattern was checked by repeating each reaction at least twice without deliberate alteration in the protocol. Although a number of tickness-diagnostic RAPD bands were noted, most of them were either rather faint or not repeatedly found in all the representative individuals of the two bulks. Thus, a large number of potentially thick, informative RAPD bands were eliminated from consideration. In contrast, the primer T16 amplified a single, bright band of approx 1200bp from thin walnut pooling, which was absent in the thick walnut pooling (Fig. 1). This band was named T16₁₂₂₄ and was amplified from all the five individuals (Fig. 2), which was selected as putative thickness markers.



Fig. 1: The result of RAPD in thickness DNA pool and thin walnut DNA pool with primer T₁₆
(In Fig. 1, the aim band 1224 had been excised from the agarose gel)

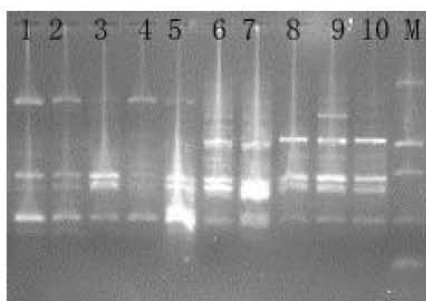


Fig. 2: The result of RAPD in individuals of both types with primer T₁₆
Note: P1 was thick walnut pool; P2 was thin walnut pool; M was PCR Marker 1~5 were thick walnut individuals; 6~10 were thin walnut individuals.

The putative thickness RAPD marker was successfully cloned and sequenced. The size of the inserted DNA fragment was confirmed by PCR analysis. The first ten nucleotides of the sequences obtained matched completely with the corresponding RAPD primers used.

The designed putatively thickness SCAR primer pair was used to amplify genomic DNAs of the 10 individuals. A single, distinct and brightly resolved band of the same size as the original RAPD fragment was obtained in thin bulk and no amplification was observed in the thick bulk. When 10 individuals of walnuts were screened with the corresponding SCAR primer pair, single bands of the expected length were amplified from each of the samples (Fig. 3).

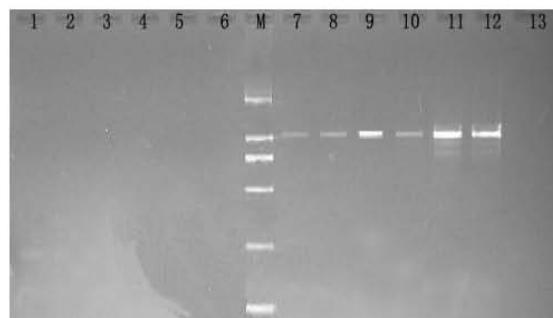


Fig. 3: PCR detection of DNA pools and their individuals of both types with specific the T16₁₂₂₄ SCAR primers
Note: 1 was thick walnut pool; 7 was thin walnut pool; M was PCR Marker 2~6 were thick walnut individuals; 8~13 were thin walnut individuals

In this investigation one thickness SCAR marker T16₁₂₂₄ was developed by designing primers from sequenced, putatively thickness, RAPD band. Inconsistencies and lack of reproducibility for amplified product profiles of RAPD reactions in independent laboratories have made RAPD markers less useful than anticipated. Thickness marker development for assistant selection in walnut breeding require reliable, reproducible, amplified genomic sequences, especially when they are to be in germplasm patent and disclosures and in legal issues of varietals infringement. The sensitivity of the RAPD reaction to a number of reaction parameters at a low annealing temperature has failed to generate consistent profiles even under laboratory conditions, which necessitates designing SCAR primers from polymorphic RAPD bands.

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