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Development of a Female-associated SCAR Marker in *Schisandra nigra* Max.

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Schisandra nigra Max., a dioecious plant native to Jeju Island in Korea, is cultivated on a small scale for fruit production. As fruit-producing female individuals are generally considered to be more valuable than male, early identification of plant sex at the seedling stage is important. In this study, a sequence-characterized amplified region (SCAR) marker associated with a female-specific region in the genome of *S. nigra* was investigated. Of 120 randomly amplified polymorphic DNA (RAPD) primers, one primer (OPB-03) consistently amplified a 749 bp band in female plants. The female-specific PCR product was isolated and cloned, and the nucleotide sequences were then determined. Southern hybridization performed using the female-specific fragment as a probe produced positive signals only in genomic DNA from the female plants. This result revealed that the 749 bp segment of DNA was present in the genome of female plants but absent in the genome of male plants. A SCAR primer pair was designed based on the RAPD marker to amplify a 436 bp fragment in the genomic DNA of female plants. This primer pair amplified the expected size of DNA fragment in female plants and four monoecious individuals collected from a natural population. The SCAR marker identified in this study can be used to distinguish female-flowering individuals at the seedling stage.

Key words : Dioecious plant, RAPD, SCAR marker, *Schisandra nigra,* sex determination

Introduction

Plants have a variety of sexual systems. More than 90% of all angiosperm species are hermaphrodites, which have perfect flowers with conserved four floral whorls; sepals, petals, stamens and carpels. However, many plant species possess a different type of sexual system, in which male and female flowers are carried either on the same or separate individuals [19]. In monoecious plants, separate male and female sexual organs are present on the same plant, whereas male and female flowers occur on separate individuals in dioecious plants. Approximately 6% of flowering species, including asparagus, kiwifruit, white campion, sorrel, hop, hemp, pepper, spinach, date palm and ginkgo etc., are dioecious [21]. A mixed condition of dioecy, in which populations are composed of hermaphrodite and one of male or female plant, and a condition of trioecy, in which male, female and hermaphrodite flowers are carried on different plants can also occur. However, these conditions are rare [1].

Schisandra nigra Max. is an endemic species in Korea that is only found in Jeju Island. This plant has been cultivated in a few orchards in Jeju Island for fruit production. *S. nigra* extract is known to contain ingredients which give a skin whitening effect via inhibition of melanogenesis [18] and promote hair growth [11].

This plant was initially thought to be dioecious. However, several monoecious plants were found from natural populations of *S. nigra* in Jeju Island. Because male plants provide no economic benefits to the grower, it is desirable for the orchard to contain many female individuals and only a few male plants. Therefore, the development of a rapid technique for the determination of sex in the seeding stage would be useful. The development of a sequence-characterized amplified region (SCAR) marker to allow identification of the sex type of *S. nigra* could also be valuable in the implementation of a breeding program for this species.

A male-specific SCAR marker of *S. nigra* was developed in previous study [10]. In this study, we cloned and sequenced a female-specific randomly amplified polymorphic DNA (RAPD) marker to design SCAR primers for the amplification of a DNA fragment found only in female plants.

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Materials and Methods

Plant materials and genomic DNA extraction

Leaf samples from male and female plants of *S. nigra* were obtained from natural populations present at Mt. Halla in Jeju Island, Korea. Four monoecious plants found in natural habitats were included in the study. Genomic DNA was extracted from the leaves using a DNeasy plant mini kit (Qiagen, USA) according to manufacturer's instructions. The DNA concentration of each sample was then determined using a NanoDrop 2,000 spectrophotometer (Thermo Fisher Scientific, USA).

PCR amplification of the female-specific fragment

A total of 120 random 10-mer primers (Operon, USA) were used for RAPD-PCR following the method described by William *et al*. [27]. PCR was carried out in 25 µl volume containing 20 ng of genomic DNA, 1.0 µM of primer, 1.25 U *Taq* DNA polymerase (Bioneer, Korea), 200 µM dNTPs, 10 mM Tris-HCl (pH 8.3), 50 mM KCl and 2.5 mM $MgCl_2$. The amplification was performed using PCR Thermal Cycler Dice (Takara Bio Inc., Japan) with the following program: 5 min initial denaturation step at 94℃, followed by 40 cycles of denaturation for 30 sec at 94℃, annealing for 30 sec at 37℃, extension at 72℃ for 30 sec, and a final extension step at 72℃ for 5 min. The RAPD-PCR products were then analyzed by electrophoresis through 1.2% agarose gels.

Cloning and sequencing of RAPD products

Following RAPD-PCR, a female-specific band was excised from the 1.2% agarose gel, and the DNA was then purified using an AccuPrep gel purification kit (Bioneer, Korea). Next, the fragment was ligated into the pGEM-T Easy vector (Promega, USA). The recombinant plasmid was transformed into *Escherichia coli* JM109 cell. Transformants with insert were identified as white colonies on LB medium containing ampicillin, isopropyl-β-D-thiogalactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) after incubating overnight at 37℃. Plasmid DNA containing the correct insert was then isolated using a Dyne plasmid miniprep kit (Dyne Bio, Korea) following the procedure recommended by the manufacturer. The nucleotide sequence of the inserted fragment was determined by SolGent Co. (Korea) using T7 and SP6 as forward and reverse primers, respectively. Sequence homologies were then examined using the BLAST program in the GenBank databases.

Hybridization analysis

The recombinant plasmid was digested with *Eco*RI and electrophoresed on a 0.8% agarose gel. The insert fragment present in the recombinant plasmid was excised from the gel and purified as described above. DNA fragment was then labeled using a DIG DNA labeling kit (Roche, Germany). Genomic DNA samples isolated from the leaves of three male and three female plants were separately digested with *Eco*RI and *Hin*dIII, and then fractionated on a 0.8% agarose gel. Next, the fractionated samples were transferred to a nylon membrane filter Hybond-N^+ (Amersham Biosciences, UK) with 0.5 N NaOH transfer buffer, and then fixed by baking for 1 hr at 80℃. The filter was hybridized with the DIG-labeled probe in hybridization buffer consisting of 5x SSC, 0.1% N-lauroylsarcosine, 0.02% SDS, and 1% blocking agent for 16 hr at 68℃. The membrane was then washed three times in buffer containing 2x SSC and 1% SDS at room temperature and DIG-labeled DNA was detected using DIG DNA detection kit (Roche, Germany) according to the manufacturer's instructions.

Design of SCAR primers and analysis

The primer set specific for female plants of *S. nigra* was designed with the Primer 3 software based on the sequences of the cloned RAPD product. The designed SCAR primers were employed for the identification of sexual type of *S. nigra* with four male, four female and four monoecious plants that were not used for RAPD and Southern hybridization. PCR was conducted under the same conditions that were used for the RAPD analysis. However, the annealing temperature used was 55℃ instead of 37℃. To test the specificity of the male-specific SCAR primers [10] on monoecious plants, amplifications were conducted under the same conditions using a male-specific primer pair (male-forward, 5′-GGAATTGAGTTCACCTCATC-3′; male-reverse, 5′-CACTT GGTCTCTTGAGTTCC-3′). The PCR products were then analyzed by electrophoresis on a 1.2% agarose gel.

Results and Discussion

RAPD amplification and cloning

One hundred and twenty random 10-mer oligonucleotides were tested to produce female-specific RAPD markers. Of these primers, only primer OPB-03 (5′-CATCCCCCTG-3′) consistently amplified a 749 bp band in the female plants. The RAPD pattern produced using primer OPB-03 is pre-

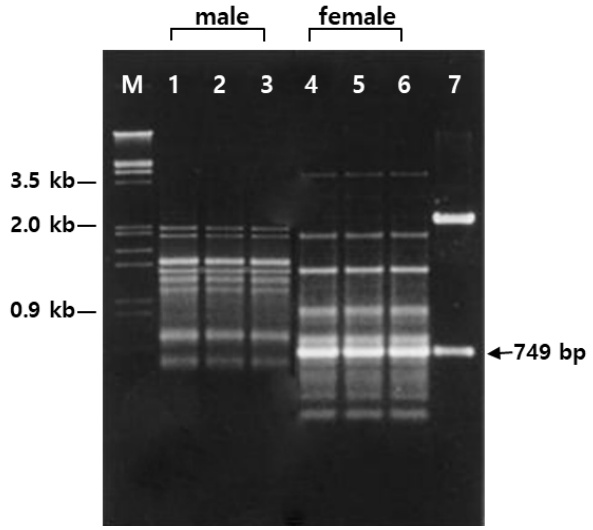

Fig. 1. Identification of female-specific RAPD marker. DNA samples obtained from individuals of male and female were amplified by PCR with the primer OPB-03. Lane M, molecular size markers; lane 1-3, male plants of *S. nigra*; lane 4-6, female plants of *S. nigra*; lane 7, DNA fragments of recombinant plasmid digested with *Eco*RI.

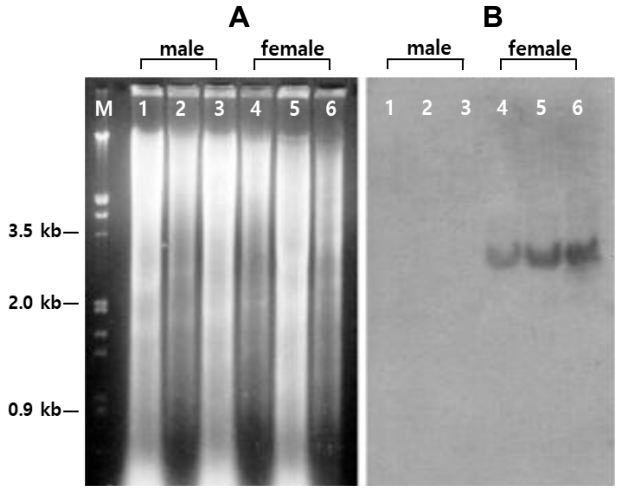

Fig. 2. Southern hybridization of male and female plants DNA with a female specific probe. (A) Electrophoresis of *S. nigra* DNA digested with *Eco*RI and *Hin*dIII through a 0.8% agarose gel. (B) Results of Southern-blot analysis. Lane M, molecular size markers; lane 1-3, male plants; lane 4-6, female plants.

sented in Fig. 1. This female-specific fragment amplified was subsequently excised from the agarose gel, purified and cloned into a T vector. The cloned fragment could be recovered by digestion of the recombinant plasmid with *Eco*RI (Fig. 1, lane 7).

Hybridization analysis

The cloned marker isolated from the agarose gel was DIG-labeled and used as a probe for hybridization. The hybridization produced about 3.0 kb of positive signals that were only seen in the lanes corresponding to female plants (Fig. 2). There was no hybridization signal in genomic DNA from male plants. These results indicate that the RAPD band produced with primer OPB-03 is a female-specific DNA fragment.

Nucleotide sequence of RAPD marker

The nucleotide sequence of the cloned fragment consisted of 749 bp that did not have a long open reading frame (Fig. 3). Homology search revealed no significant similarity at nucleotide level to any sequences in the database.

Development of the female-specific SCAR maker

To establish a rapid and reliable PCR-based technique for sex determination of *S. nigra*, the female-specific RAPD marker was converted into a pair of SCAR primers based on its nucleotide sequence. The following 20-nucleotide PCR primers were designed from the internal region of the RAPD marker: 5′-CTTGTTGCCCTATTCTTCAC-3′ (female-forward) and 5′-AACACCAGCTACTCTCTCCA-3′ (female-reverse) (Fig. 3, shaded and underlined). This primer set was expected to amplify a 436-bp fragment. The specificity of the primer set was tested using genomic DNAs obtained from four male, four female and four monoecious plants. PCR performed using this primer set amplified 436-bp fragments when DNAs obtained from female and monoecious individuals were used as a template; however, this primer set did not cause amplification with DNA obtained from male plants (Fig. 4A). Additionally, male-specific primers [10] produced 459-bp fragments when DNAs obtained from male and monoecious plants were used as a template (Fig. 4B). In monoecious plants, both male and female flowers bloom in the same individual. As shown in Fig. 4, DNA fragments of the expected size were amplified with male- and female-specific primer pairs, respectively, in the monoecious plants. These results suggest that the identified sex-specific DNA fragments may be related to the development of the reproductive organs in *S. nigar*.

The mechanisms used to determine the sexual phenotype in plants are diverse, ranging from genetic control to epigenetic or hormonal regulation [25]. Although the first sex chromosomes were discovered in dioecious plants in the early 20th century [3], our understanding of the basis of sex

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                10                  20                  30                  40                  50                  60
5′  GGACTGCAGA      CATCGATTAC      ATAACGAAAA      TCACCATAGA      AACCTCTCTT      TCTTATAGGC
               70                  80                  90                 100                 110                 120
    GTACAAAGAG      GGTCCCATAG      ATCAGGATAT      AGGTTGAGAA      GCACTACGAA      GGACTACCGG
              130                 140                 150                 160                 170                 180
    CAAAAGAGTA      AATCATCGAT      CGACTCAATT      CTTTCCTATC      TTGGTCTAGA      AAGAGAGGCG
              190                 200                 210                 220                 230                 240
    CTGAAFTAAC      TGGCAATAGT      GCAGAAAAAA      GCTTTACCAT      CAAGCCGCTT      GCTTTTGTCT
              250                 260                 270                 280                 290                 300
    CTGCCTTCTT      TTGTGAGAAT      TTATCGGTAC      CGTACACCAC      CTGAAGCGGA      GTCGGAGCTT
              310                 320                 330                 340                 350                 360
    GTTGCCCTAT      TCTTCACTGA      GCACAGTGGA      CTGACTTTGC      TTTGTATGAT      TTTTCCTTGC
              370                 380                 390                 400                 410                 420
    CCAAGACTGA      CATTTCGTTC      GTTTGAGTGC      ATGAACTGCT      GTGTTAAGTA      TGTGTAAACT
              430                 440                 450                 460                 470                 480
    TAAAAATAAA      AGTAGATGGT      TGATGTTAGA      GAAGTCCCTT      CTATGATTCG      TTACCGAATC
              490                 500                 510                 520                 530                 540
    GTATGCCTTT      GAGTTGTTTT      GCTTTCACAA      GACAAGGGGA      TGTGCTCCCA      TCCCTCCGGA
              550                 560                 570                 580                 590                 600
    GATCATAGGA      AAGATGGAGT      GATTCTCCAT      CTGGCTTCTA      CCTATTTAAG      AGAGTCGATA
              610                 620                 630                 640                 650                 660
    TTTCACTTTA      AAGACAGCTC      GTGGCCTAGC      TACCAGATAG      AATACAGTCT      TACCTGTCCT
              670                 680                 690                 700                 710                 720
    AGCTTGATAG      AAACCTAGCT      ACAAAAGCGA      CTTCCCCCTG      TTGAAAATAC      CAGTGGAGAG
              730                 740
    AGTAGCTGGT      GTTGCTTACT      CTGCAGTCC   3′
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Fig. 3. Nucleotide sequences of the female-specific RAPD marker. The female-specific SCAR primers are shaded and underlined. This primer set was designed to amplify a 436 bp fragment internal to the 749 bp of the RAPD marker.

determination and the structure of sex chromosomes is poor. Because sex determination in many dioecious plants occurs via sex-specific activation and reduction of cell division in the primordia of stamens and pistils, flowers from male plant cannot be distinguished from female flowers during their early development [5, 15, 16]. Therefore, the discovery of a sex-specific DNA fragment is an important step in identifying the sex of a plant during its early developmental stage. Sex-specific DNA fragments can also be useful for studying the evolution of sex chromosomes in plants.

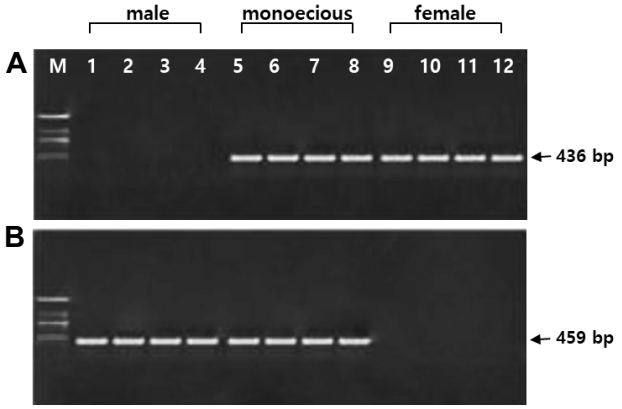

Fig. 4. Validation of sex-specific SCAR markers. Female- and male-specific primers were used for PCR with genomic DNA of *S. nigra*. The female primers amplified a fragment of 436 bp in female and monoecious plants (A), while the male primers produced a 459 bp in male and monoecious plants (B). Lane M, size marker, 100 bp ladder; lane 1-4, male plants; lane 5-8, monoecious plants; lane 9-12, female plants.

Molecular markers that are tightly linked to a plants sex have been reported for various dioecious species [6]. RAPD has been used to identify markers specific to sex, and the products of the RAPD analyses have been converted into stable and reliable SCAR primers for many dioecious plant species, including *Asparagus officinalis* [9], *Atriplex garrettii* [22], *Cannabis sativa* [13, 23], *Carica papaya* [20], *Ginkgo biloba* [12], *Phoenix dactylifera* [2], *Piper longum* [14], *Pistacia vera* [8], *Rumex nivalis* [24], *Salix viminalis* [7] and *Silene latifolia* [17].

S. nigra has three sex types, male, female and monoecious. Because this type of breeding system is unique and relatively rare in flowering plants, this species may provide valuable insight into the mechanism and evolution of plant sex. To date, no known studies regarding the molecular mechanism controlling sex determination or chromosome number in this plant have been conducted. In papaya, three sex types, male, female and hermaphrodite, are determined by a sex chromosome system with two slightly different Y chromosome [28]. Molecular markers tightly linked to the gene that determines the sex of papaya have been developed [4, 26]. Because the *S. nigra* population contains male, female and monoecious plants, it can be postulated that this plant has a sex determination system similar to that of papaya. As shown in Fig. 4, female- and male-associated SCAR primers amplified PCR products exclusively in monoecious and female or male plants. The recovery of sex-associated DNA fragments from *S. nigra* suggests that sex determination is genetic in this

species, and that male and female SCAR markers might be located on the regions of the chromosome that are specific to each sex type.

The results presented here indicate that it is possible to determine the sex of *S. nigra* plants accurately and rapidly using a PCR-based technique. Male- and female- specific markers can also be used as starting points to reveal sex-associated genes present on the chromosomes.

The Conflict of Interest Statement

The authors declare that they have no conflicts of interest with the contents of this article.

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초록 : *Schisandra nigra* Max.에서 암그루에 연관된 SCAR 마커의 개발

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자웅이주 식물로 알려진 *Schisandra nigra* Max. (흑오미자)는 우리나라 제주도에 자생하고 있으며, 열매를 얻기 위해 일부 농가에서 재배되고 있다. 열매 생산을 위해서는 수그루에 비해 암그루의 가치가 더 높기 때문에 묘목 단계에서 일찍 성별을 아는 것은 중요하다. 이 연구에서는 *S. nigra*의 유전체에서 암그루에 특이적인 부위에 관련된 SCAR 마커를 개발하였다. 120개의 무작위로 구성된 RAPD 프라이머 중에서 OPB-03 프라이머가 암그루에서 749 bp의 밴드를 안정적으로 증폭시켰다. 암그루 특이적인 PCR 산물을 분리하여 클로닝한 뒤 염기서열을 결정하였다. 이 암그루 특이적인 절편을 탐침으로 사용한 Southern hybridization에서 암그루에서만 양성반응이 나타나고 수그루에서는 잡종화가 일어나지 않았다. 이러한 결과는 749 bp의 DNA 절편이 암그루의 유전체에는 존재하지만, 수그루에는 없음을 시사하였다. RAPD 마커로부터 암그루에서만 436 bp를 증폭시키는 SCAR 프라이머를 설계하였다. 이 프라이머 쌍은 암그루와 자생지에서 수집한 4개의 자웅동주 식물에서만 예상되었던 크기의 DNA 절편을 증폭하였다. 이 연구에서 개발된 SCAR 마커는 묘목 단계에서 암꽃이 피는 개체를 선발하는데 사용될 수 있을 것이다.