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A highly reliable DNA marker in dioecious plant Japanese hop (*Humulus scandens*)

WUJUN GAO[1], YANKE JI[2], CHUANLIANG DENG[3] and LONGDOU LU[4]

Henan Normal University, Xinxiang, Henan, China 453 007

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ABSTRACT

One hundred decamer primers of random amplified polymorphic DNA were tested on dioecious *Humulus scandens* plants to analysis genome difference. One of the 100 primers, S2142, produced one male-linked band ($S2142_{762}$), with the length of 762bp, rich in AT (54.7%). The marker was sequenced and specific primer was constructed to generate correspondingly size marker of sequence characterized amplified regions (SCAR) in male plants, $SCAR_{762}$, which is suitable for a precise, rapid identification of male plants in 30 male and female *H. scandens* plants, respectively. Southern hybridization, using $S2142_{762}$ as a probe, showed that $S2142_{762}$ exists in the male genomes, but not in the female genome. This result strongly suggests that $S2142_{762}$ is located on the chromosome region that is specific to the male.

Key words: Dioecious, *Humulus scandens*, Male linked, RAPD, SCAR

The majority of flowering plants are bisexual. Only about 6% of angiosperms are dioecious, where individuals either produce staminate or pistillate flowers for example, *Carica papaya*, *Actinidia deliciosa*, *Asparagus officinalis*, *Silene latifolia*, and *Cannabis satica* (Jamilena *et al.* 2008). Moreover, the independent evolutionary origin sex-determination in plants is still at primary stage, compared with the ancient origin in animals. Dioecious plants should be an appropriate working model for studying primitive mechanisms of sex determination and differentiation to explore the origin of sexual dimorphism.

Humulus scandens, dioecious, is a twining or prostrate vine that grows as an annual in north-eastern China and as a perennial in southern China (Guo *et al.* 2009). Male and female plants are only be distinguished morphologically by either their flowers or flowers buds. *H. scandens* is determined by heteromorphic sex chomosomes. Males bear both X and Y chromosomes ($2n=14+XY_1Y_2$), females bear a pair of X chromosomes (2n=14+XX) (Wang *et al.* 2007). In addition, the genetic control of sex seems stricter in *H. Scandens.* It has not proved possible to reverse sex in *H. scandens* either by the exogenous application of growth regulators (Khryanin 2002). *H. scandens* should be useful for examining the origin of sex chromosome with respect to the molecular mechanism of sex expression.

Compared to other molecular markers, such as inter-simple sequence repeat (ISSR), amplication fragment length polymorphism (AFLP), RAPD markers have an advantage because they are easily generated and especially suitable for genome different study in plant species whose detailed genomic sequence information is not available. The limitation of RAPD markers is their variation between DNA preparations and assay conditions are not very reliable. However, RAPD can be converted into stable and reliable markers by cloning the amplified bands, sequencing the ends, and using the sequences to generate extended oligomer primers. These extended oligomers, when annealed under stringent by hybridization conditions, reproduce the amplification of single bands, corresponding to genetically defined loci, sequence characterized amplified regions (SCAR). This approach has been employed to develop several sex-linked molecular markers of dioecious plants including *Asparagus officinalis* (Gao *et al.* 2007), *Pistacia vera* (Yakubov *et al.* 2005), *Cannabis sativa* (Otto *et al.* 2002), *Carica papaya* (Urasaki *et al.* 2002), *Salix viminalis* (Gunter *et al.* 2003), *Rumex acetosa* (Helena 2002), *Mercurialis annua* (Khadka *et al.* 2002) and *Eucommia ulmoides* (Xu *et al.* 2004).

Previously, some chromosome and biochemical marker linked to sex were developed in *H. scandens* (Wang *et al.* 2007). But no information about the molecular mechanism of sex determination was reported. Here, we present the

[1]Associate Professor (e mail: gaowujun@yahoo.cn), [2]Graduate Student (e mail: william21sj@sohu.com), [3]Associate Professor (e mail: dcl75@163.com), [4]Professor (e mail: lld5910 @yahoo.com), College of Life Science

development of a sex-linked SCAR marker in *H. scandens* by combining RAPD with bulk segregant analysis (BSA) of female and male DNA pools.

MATERIALS AND METHODS

H. scandens plants were grown under natural light and photoperiod in an experimental field near the Henan Normal University during 2006–10. After determining the sex types of the plants, individual leaf samples were collected (about 1.5 g) and stored a –80°C.

Genomic DNA was extracted separately from each sample using the cetyltrimethyl ammonium bromide (CTAB) method (Xiao *et al.* 2008) with minor modifications. About 0.3 g of frozen leaf tissue was ground to a fine powder in liquid nitrogen and mixed with 700 µl of CTAB extraction buffer (100 mM Tris-HCl (*p*H 8.0), 1.4 M NaCl, 20 mM EDTA (*p*H 8.0), 2% CTAB, 0.5% $NaHSO_3$, 1% ß-mercaptoethanol, 1% polyvinylpyrrolidone) in a 1.5 ml Eppendorf tube. The mixture was incubated at 65°C for 1 hr. Then an equal volume of chloroform: isoamyl alcohol mixture (24: 1) was added, and centrifuged at 10 000 g for 10 min. The aqueous phase was repeatedly decanted and transferred to a fresh tube to reduce impurity between two phases. Extraction was performed twice using phenol: chloroform: isoamyl alcohol (25: 24: 1) mixture and chloroform: isoamyl alcohol mixture (24: 1), respectively. The last aqueous phase was mixed with 2/3 of isopropanol and 1/10 of 2 M sodium acetate (*p*H 5.2) at –20°C for at least 1 hr to precipitate DNA and centrifuged at 8 000 g for 15 min at 4°C. The nucleic acid precipitate was washed with 70% ethanol, airdried and suspended in 200 µl of double distilled water containing 10 mg/ml RNAase at 37°C for 1 hr. The isolated DNA was diluted in double distilled water to a final concentration of 40 ng/µl and subjected to polymerase chain reaction (PCR) amplification. Two bulks of DNA samples were made by pooling an equal amount of DNA from 10 randomly selected individuals, each for the male and female plants.

RAPD analysis was performed with a set of 10 mer 100 random primers (Shanghai Sangon, China) in 25 µl volume reaction mixture, which containing 10 ng genomic DNA, 10 mM Tris-HCL (*p*H 9.0 at 25°C), 50 mM KCl, 0.5 mM $MgCl_2$, 0.2 mM deoxyribonucleoside triphosphates, 0.5 µM primer, and 0.75 unit of Taq DNA polymerase. DNA amplification was performed in Gene Amp PCR System 2400 thermal cycler (Perkin-Elmer Corporation, United States) with the following cycling parameters: 45 cycles of 30 sec. at 94°C, 45 sec. at 36°C and 90 sec. at 72°C, followed by one cycle of 7 min. at 72°C and then hold at 4°C prior to analysis. Amplification products were analysed by gel electrophoresis in 1.5% agarose gel (Promega, United States) in 1×TAE (0.04 M Tris-acetic acid, 0.01 M EDTA) buffer. Gels were stained with ethidium bromide and visualized in a UV light. Each amplification reaction was performed using a single primer and repeated at least three times in order to verify the reproducibility of the results.

The candidate RAPD fragment, which present in male and absent of in female plants, was excised from the gel using UNIQ kit (Shanghai Sangon, China), and then cloned into the pGEM-T vector (Shanghai Sangon, China). The recombinant vectors were transformed to *Escherichia coli* JM109 cells (Dingguo, China). The cells were then spread on LB medium containingampicillin (50µg/ml), isopropyl-ß-D- thiogalactopyranoside (0.95 µg/ml), and 5-bromo-4-chloro-3-indoly-ß-D-galactopyranoside (X-gal) (40µg/ml), and incubated at 37°C overnight. Transformants with inserts were identified as white colonies. Plasmid minipreps were prepared from overnight cultures using kit (Shanghai Sangon, China). The inserted RAPD fragment in the plasmid was sequenced by dideoxynucleotide chain termination method using T7 and SP6 as forward and reverse primers, respectively. The RAPD fragment sequence was used as query to do BLAST (NCBI, National Center for Biotechnology Information, United States) to confirm if it shares any significant homology with any of the sequences at GenBank databases.

The ends of the cloned RAPD fragment sequences were used to design 24-bp SCAR primers for amplification of the fragment from genomic DNA. Amplification of genomic DNA with SCAR primers was performed by using one cycle of 5 min. at 94°C; 30 cycles of 30 sec. at 94°C, 45 sec. at 60°C/64°C/65°C, 90 sec. at 72°C; and one cycle of 7 min. at 72°C. The amplification products were separated in 1.5% agarose gel. The 24-bp SCAR primer sequences are listed in Table 1.

One male and one female plants were crossed to generate 11 F_1 plants. A population of 143 F_2 plants from a cross between F_1 plants cross each other. This population consisted of 61 male and 82 females plants. Genomic DNA was extracted from individual plant. SCAR marker was amplified from the genome DNA of each plant using the SCAR primer.

Genomic DNAs isolated from young leaves of male and female plants were separately digested with *Eco*RI and *Hind* III. The digests were fractionated on a 1% agarose gel and

Table 1 Primer of SCAR marker linked to sex locus in *H. scandens*. Each primer contained the original 10-bp RAPD primer plus the first 14-bp from each end

RAPD primer	SCAR primer	SCAR primer sequence	Annealing
S2142	SCAR2142F	GTAAGCCGAG CTTGGGTTAA CCAC	58
	SCAR2142R	GTAAGCCGAG GATAATGATT GATG	

transferred to a nylon membrane filter (Hybond-N$^+$, Amersham Pharmacia Biotech, United States) with 0.5 M NaOH transfer buffer. The filters were hybridized with the ^{32}P-labelled S2142$_{762}$ probe at 65°C in hybridization buffer consisting of 5×SSC, 5×Denhardt's (1% Ficoll, 1% polyvinylpyrrolidone, 1% BSA) solution and 0.5% SDS. Washing conditions were as follows: two washings in low stringency buffer (2×SSC containing 0.1% SDS) at 65°C for 25 min, and two washings in high stringency buffer (0.1×SSC containing 0.1% SDS) at 65°C for 30 min. The filters were exposed to an imaging plate.

RESULTS AND DISCUSSION

PCR amplification

To identify genome different and sex linked marker in *H. scandens*, a total of 100 random decamer primers giving approximately 1,500 bands were used to screen out the DNA markers linked to sex. The primer S2142, with the sequence 5'- GTAAGCCGAG-3', generated the band S2142$_{762}$, which was present only in male and absent in female plant. (Fig. 1a). The fragments was cloned into the pUCm-T vector. In order to verify the reliability of the RAPD fragments, the marker PCR amplification was performed in 10 male and female plants, respectively. The resulted showed that band S2142$_{762}$ was generated in all 10 male individuals while none of the females.

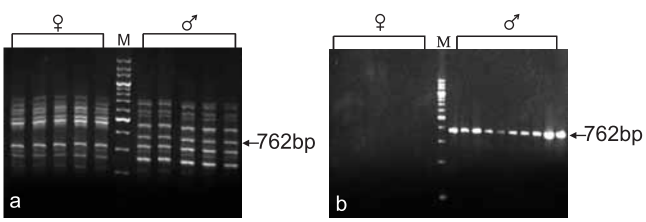

Fig 1 Electrophoresis of male-linked DNA fragments (a) and SCAR$_{762}$ (b) markers produced by PCR with S2142 primer and SCAR primers on 1.5% agarose gel. M-molecular size markers

Sequence analysis of sex-linked fragment

The cloned fragment amplified by the primer S2142 was sequenced. The fragment is 762 bp in length and contains 54.7% of A+T (Genbank accession no. EU882085). Sequence analysis indicated that the fragments had no any recognized open reading frame. Homology searching revealed no significant similarity at nuclotide sequence level to any sequence in the database. Only limited homologies (70% identity) were found with the repeated regions of LTR retrotransposon sequences (98bp), suggesting S2142$_{762}$ sequence might be in a non coding region of the genome.

Conversion of male-linked markers to SCAR markers

To establish reliable PCR-based technique for *H. scandens* sex determination, the RAPD marker S2142$_{762}$ was converted to SCAR marker. Based on the sequence of S2142$_{762}$, a pair

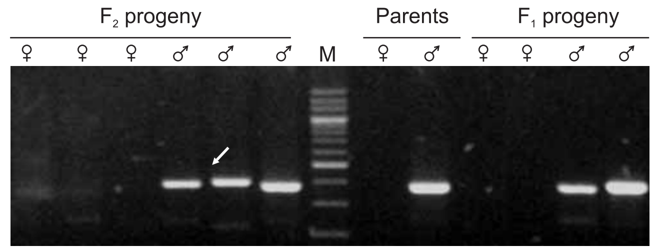

Fig 2 Electrophoresis of male-specific SCAR$_{762}$ markers of mapping population by PCR with SCAR primers on 1.5% agarose gel. M-molecular size markers. Arrow indicted a 790bp band

of strand-specific 24-bp primer pair was designed (Table.1) converting the RAPD marker into single-locus PCR marker sequence characteristic for amplified region SCAR marker. PCR performed with these primers amplified expected DNA fragment only in the male individuals (Figs 1, 2, 3). This suggests that the specific DNA region of S2142$_{762}$ exists in the male plants, but not in the female plants, which was confirmed by repeated experiments with a large population of *H. scandens* plants (data not shown). Presence or absence of S2142$_{762}$ in our RAPD experiments was not caused by base changes at the primer-binding sites. Thus, SCAR$_{762}$ converted from RAPD markers is reliable genome different sequence between male and female of *H. scandens* plants.

Sex identification assay based on mapping population

To identified the stability of SCAR$_{762}$ in the mapping population. SCAR$_{762}$ was amplified from genome DNA of population of parents, F$_1$ and F$_2$ progeny. Four male present the SCAR$_{762}$ and seven female plants absent of the marker in all 11 F$_1$ individuals. Eighty-one of eighty-two female plants absent of the SCAR$_{762}$ and one female plant present the SCAR marker in F$_2$ population (Fig 2). Sixty male plants present the SCAR marker in all sixty-one plants. It's worth noting that a comparatively large size band (790bp) was amplified from two male plants (Fig 2), which imply that the sequence of SCAR$_{762}$ happen to various by inserting.

Southern blot analysis

Southern hybridization was carried out with genomic DNAs of the male and female plants of *H. scandens*, using a 32P-labeled S2142$_{762}$ fragment as the probe. Hybridization to *Eco*RI- and *Hind* III- digested DNAs showed bands in the male plants, but not in female plants (Fig 3). This suggests that S2142$_{762}$ may be located in the chromosome region that is specific to male sexes indicating the possibility of chromosomal differentiation between sexes. In addition, cytological studies have success to obtain heteromorphic sex chromosomes (Wang *et al.* 2007).

The method of genomic DNA extraction from *H. scandens* is rarely reported. Based on our previous the DNA extraction method for *H. Scandens* (Xiao *et al.* 2008), a simple method of genomic DNA extraction from the *H. scandens* is

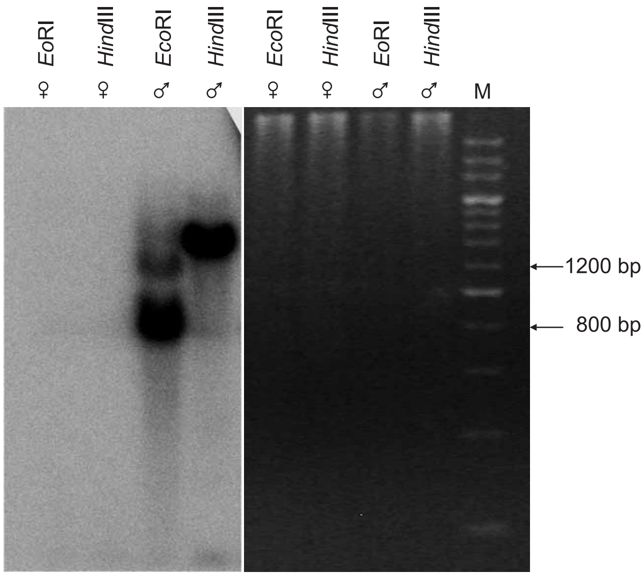

Fig 3 Southern-blot analysis of $S2142_{762}$ marker. Genomic DNA from male and female plants of *H. scandens* was restricted with different enzymes and hybridized with ^{32}P-labeled $S2142_{762}$ probe.

established in this study, and the extracted DNA has proved to be good for further studies.

RAPD is regarded as a preferred method to SSR or AFLP analysis despite its disadvantages of being unrepeatable and unstable. Many studies have been carried out to improve RAPD repeatability and stably. In the absence of genetic information on sex determination in dioecious plant, the use of molecular markers for discriminating between male and female genome is worthwhile (Khadka *et al.* 2002; Peil *et al.* 2003; Gao *et al.* 2007). Here, we describe the development of new PCR-based markers highly linked to sex in *H. scandens*. The RAPD markers were found always associated with the male genotype. The marker identified here was proved to be reproducible under various amplification conditions. The results still remained reproducible even if the marker was tested by various Taq polymerases and thermal cycles. Moreover, we successfully converted this RAPD marker to SCAR marker, which was verified by predicting correctly the sexes of 30 *H. scandens* plants from various populations with $SCAR_{762}$. SCAR markers are more reliable because they can detect only a single locus and their amplification is less sensitive to reaction conditions. These characteristics suggest that the SCAR marker male-linked in *H. Scandens* ($SCAR_{762}$) plants could be located on sex chromosomes.

It is believed that sex chromosome derived from a pair of autosomes. Recombination suppression lead to the sex chromosome differentiated around the sex determination locus (Ray and Moore 2007, Jamilena *et al.* 2008). The suppression of recombination between proto-X and proto-Y predicts an accumulation of some non-coding sequence, such as $SCAR_{762}$, including transposable elements (Liu *et al.* 2004, Katsuyuki *et al.* 2007), retrotransposon (Sakamoto *et al.* 2000) and repetitive seqence (Stehlik and Blattner 2004, Ray and Moore 2007, Mariotti *et al.* 2009), which lead to sex chromosomes enlargement to possess some sepecific functions. In contrast to the large-scale sequence and the separation of function genes, molecule markers provide a better clue to the differences between the male and the female genome.

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