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A male and hermaphrodite specific RAPD marker for papaya (*Carica papaya* L.)

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Abstract The random amplified polymorphic DNA (RAPD) technique was used to determine the sex of a dioecious species, *Carica papaya* L., with three sex types, male, female and hermaphrodite. A 450 bp marker fragment, named PSDM(Papaya Sex Determination Marker), exists in all male and hermaphrodite plants but not in the female plants so far analyzed. The DNA sequence of PSDM exhibited no significant similarity to previously reported sequences. A sequence-characterized amplified region (SCAR) marker, SCARps, was developed from PSDM to determine the sex of papaya. Southern hybridization, using PSDM as a probe, showed that PSDM exists in the male and hermaphrodite genomes, but not in the female genome. This result strongly suggests that PSDM is located on the chromosome region that is specific to the male and the hermaphrodite. SCARps is a suitable marker for the precise and rapid diagnosis of sex in papaya.

Keywords RAPD · Sex determination · *Carica papaya* · Dioecious plant · SCAR marker

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Introduction

Papaya, *Carica papaya* L., which originated in tropical America, provides economically important edible fruits and latex in tropical and subtropical areas. For instance, papain, a thiol protease abundant in the milky latex of fruits, is used for food and for the textile and perfume industries (Jones and Mercier 1976). Papaya is a polygamous diploid (2n=18) plant species with three sex types, i.e. male, female and hermaphrodite. Male plants are useless for economic purposes. As a food, pyriform fruits from hermaphrodite trees are preferred on the market over spherical fruits from female plants. It is therefore desirable that only hermaphrodite individuals are cultivated in the field. However, as sex determination of papaya plants at the seedling stage was not possible, papaya cultivation has been commonly carried out by planting 2–3 seedlings in a pit, followed by the uprooting of undesired female plants after flowering, which entailed space and labor cost. Thus the development of a rapid technique for papaya sex identification is necessary.

Genetic analysis of papaya sex determination was carried out by crossing individuals of different sex types (Storey 1941). Crossing of female and male plants resulted in a 1:1 ratio of the female and male progeny. Likewise, a cross between female and hermaphrodite plants resulted in a 1:1 ratio of the female and hermaphrodite progeny. A cross between the hermaphrodite and the male gave rise to male, hermaphrodite and female offspring in a 1:1:1 ratio. The selfing of hermaphrodite plants produced hermaphrodite and female offspring in a 2:1 ratio. From these observations, Storey (1953) hypothesized that papaya sex is determined by three alleles, *M, H* and *f*, at a single locus *Sex1*. The alleles *M* and *H* were assumed to be dominant over the *f* allele. Thus, the male, hermaphrodite and female sexes are determined by the *Sex1* locus genotypes, *Mf, Hf* and *ff*, respectively. Homozygotes of dominant alleles (*MM* and *HH*) as well as a heterozygote (*MH*) were assumed to be lethal. Cytological trials to identify heteromorphic chromosomes were not successful (Hofmeyr 1939).

Recently, molecular markers tightly linked to the sex of dioecious plants have been reported. For example, AFLP (amplified fragment length polymorphism) markers were developed for asparagus (Reamon-Büttner et al. 1998; Reamon-Büttner and Jung 2000), and for *Dioscorea tokoro* (Terauchi and Kahl 1999), and RAPD (random amplified polymorphic DNA, Williams et al. 1990) markers for *Pistacia vera* (Hormaza et al. 1994), *Cannabis sativa* L. (Sakamoto et al. 1995; Mandolino et al. 1999), asparagus (Jiang and Sink 1997) and *Silene dioica* (Di Stilio et al. 1998). In papaya, RAPD and microsatellite markers linked to sex have been reported (Sondur et al. 1996; Parasnis et al. 1999). Two RAPD markers, *T12* and *T1C*, were each mapped 7-cM apart from the *SEX1* locus (Sondur et al. 1996). In a Southern hybridization study using the oligo- nucleotide $(GATA)_4$ as a probe, Parasnis et al. (1999) identified sex-linked DNA fragments. However, a papaya DNA marker both tightly linked to sex and easy to score has not been available so far.

In this paper, we report a RAPD marker specific to male and hermaphrodite plants. Its conversion into a SCAR (sequence-characterized amplified region) marker allowed rapid sex identification in papaya. We further show evidence that this marker is located on the chromosome segment which is specific both for the male and the hermaphrodite.

Material and methods

Plant materials and genomic DNA extraction

The plant materials used in this study are shown in Table 1. They include the two commercially important Hawaiian cultivars, 'Sunrise Solo' and 'Waimanalo Solo', as well as four Okinawan landraces, IK-2, IK-7, IK-10 and TK-5 and a Mexican landrace, Me-1. Four additional lines were derived from crosses between Hawaiian, Okinawan and Mexican cultivars (ON-7, ON-12, ON-34 and ON-36 crossbred at Okinawa Prefectural Agricultural Experiment Station, Okinawa, Japan). Two breeding families were included in the study to determine the cosegregation of sex and the DNA marker in the progeny. One family consists of male parent ON-7 and female parent ON-36 and their F_1 progeny, and another family comprises hermaphrodite 'Sunrise Solo' as the male parent, ON-34 as the female parent and their F_1 progeny.

Genomic DNA was extracted from young leaves using the standard CTAB method with minor modifications (Saghai Maroof et al. 1984). DNA concentration was determined spectrophotometrically, and its integrity and concentration further checked by agarose-gel electrophoresis.

PCR amplification of the sex-specific fragment

PCR for RAPD (Williams et al. 1990) analysis was carried out in 20-µl volumes containing 100 ng of genomic DNA, 1.0 µM of IBRC-RP07 primer (5´-TTGGCACGGG-3´), 0.2 U of Ex *Taq* DNA polymerase (TaKaRa), 250 µM of dNTPs, 10 mM Tris-HCl (pH 8.3), 50 mM KCl and 1.5 mM $MgCl_2$. Reaction conditions consisted of 30 cycles of 94°C for 1 min, 37°C for 1 min and 72°C for 2 min followed by a final extension at 72°C for 5 min in a PERKIN ELMER PCR Thermal Cycler.

PCR of the SCAR (sequence-characterized amplified region) marker was carried out in 20-µl volumes containing 10 ng of genomic DNA, 1.0 µM each of primers SDP-1 (5´-GCACGATTTA-GATTAGATGT-3´); and SDP-2 (5´-GGATAGCTTGCCCAGGT-CAC-3´), 0.2 U of Ex *Taq* DNA polymerase (TaKaRa), 250 µM of dNTPs, 10 mM Tris-HCl (pH 8.3), 50 mM KCl and 1.5 mM $MgCl_2$. Amplification was performed for 30 cycles at 94°C for 1 min, 55°C for 1 min and 72°C for 1 min, followed by a final extension at 72°C for 5 min. PCR products were separated by electrophoresis in 2.0% agarose gels, and visualized by ethidium bromide staining.

Cloning and sequencing of a RAPD marker fragment, PSDM

A sex-linked marker fragment, PSDM, amplified by the IBRC-RP07 primer from male and hermaphrodite DNA, was extracted from the agarose gel and cloned into the pT7Blue vector (Novagen). Cloned fragments were sequenced by an ABI 377 DNA sequencer using the BigDye Terminator Cycle Sequencing FS kit (ABI).

Preparation of the DNA probe

The cloned fragment, PSDM, was recovered from the plasmid by double digestion with *Eco*RI and *Pst*I, and labelled with [α-^{32}P] dCTP by random priming, using RediprimeTMII (Amersham Pharmacia Biotech). The labelled probe was purified with a ProbeQuant G-50 Micro Column (Amersham Pharmacia Biotech) and used as a probe for Southern hybridization.

Southern-blot analysis

Ten micrograms of DNA each isolated from young leaves of a male plant IK-7, a female plant of cv 'Waimanalo Solo' and a hermaphrodite plant of cv 'Sunrise Solo' were separately digested with *Eco*RI and *Taq*I. The digests were fractionated on a 1.0% agarose gel and transferred to a nylon membrane filter (Hybond-N+, Amersham Pharmacia Biotech) with 0.5 N NaOH transfer buffer. The filters were hybridized with the ^{32}P-labelled PSDM probe at 65°C in hybridization buffer consisting of 5×SSC,

Table 1 Papaya cultivars analyzed

Code no.	Cultivar/line	Source
1	Waimanalo Solo	Hawaiian cultivar
2	Sunrise Solo	Hawaiian cultivar
3	IK-2	Okinawan land race (Ikema island)
4	IK-7	Okinawan land race (Ikema island)
5	IK-10	Okinawan land race (Ikema island)
6	TK-5	Okinawan land race (Tokashiki island)
7	Me-1	Introduced from Mexico
8	ON-7	Me-1 (female)×IK-7 (male)
9	ON-12	Me-1 (female)×Waimanalo Solo (hermaphrodite as a male)
10	ON-34	IK-10 (female)×Sunrise Solo (hermaphrodite as a male)
11	ON-36	TK-5 (female)×IK-10 (male)

5×Denhardt's 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 30 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, and analyzed with a Fuji BAS 2000 phospho-image analyzer.

Results and discussion

A RAPD marker PSDM specific for males and hermaphrodites

After trying 25 arbitrary 10mer primers, we identified primer IBRC-RP07 (5´-TTGGCACGGG-3´) that discriminates the sex of papaya. The first experiment (Fig. 1A) involved a total of 17 individuals (six male plants, seven female and four hermaphrodites) including the cultivars 'Sunrise Solo' and 'Waimanalo Solo', four Okinawan landraces and a Mexican landrace, for which the sex of the plants are known. We could identify a 450-bp fragment (PSDM) present in the male and hermaphrodite plants, but not in the female plants, with no failure. The second experiment (Fig. 1B) involving 14 progeny derived from a cross between male parent ON-7 and female parent ON-36 also showed the presence of the PSDM fragment in the male, but not in the female individuals. We further tested the diagnostic power of PSDM by first checking its presence/absence in 14 seedlings of F_1 progeny derived from a cross between a hermaphrodite plant of 'Sunrise Solo' and female parent ON-34, and later observing their sex after flowering (Fig. 1C). All individuals carrying the PSDM marker turned out to be hermaphrodites, and those without PSDM were shown to be females.

DNA sequence of PSDM

DNA fragments corresponding to PSDM were isolated separately from male and hermaphrodite plants, and were cloned. The sequences from the male and hermaphrodite PSDM with a size of 450 bp were identical (Fig. 2). The sequence of the arbitrary primer, IBRC-RP07, exists at the 5´ and 3´ ends of the PSDM fragment. No DNA sequence with a significant similarity to the 450 bp sequence could be identified in GENBANK.

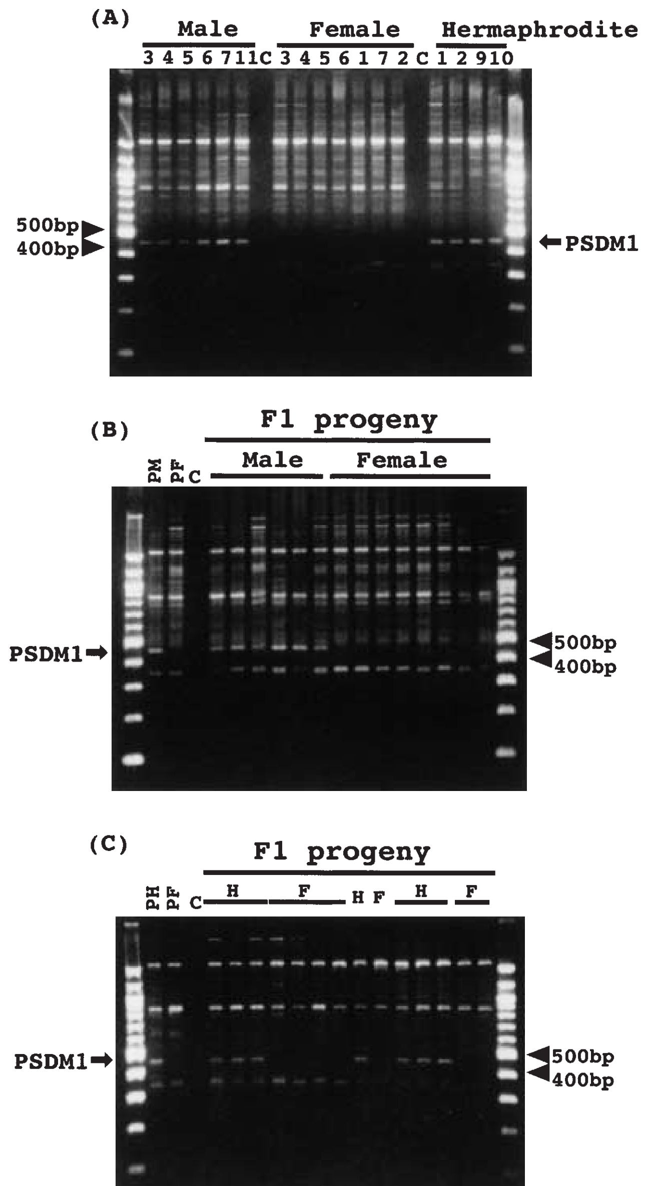

Fig. 1A–C RAPD banding patterns of male, female and hermaphrodite papaya plants obtained by the arbitrary primer IBRC-RP07. *C* no template. (**A**) Numbers correspond to the code nos. in Table 1. (**B**) *PM*, male parent ON-7; *PF*, female parent ON-36. (**C**) *PH*, hermaphrodite parent, cv 'Sunrise Solo', used as a male parent; *PF* female parent ON-34; *H* hermaphrodite plant; *F* female plant. *PSDM* (Papaya Sex Determination Marker) is a male and hermaphrodite specific marker

Conversion of PSDM to a SCAR marker

To establish a rapid and reliable PCR-based technique for papaya sex determination (Jiang and Sink 1997; Mandolino et al. 1999), the RAPD marker PSDM was converted to a SCAR marker. Two primers, SDP-1 and SDP-2, were designed from sequences within the PSDM sequence (Fig. 2). PCR performed with these primers amplified a 225 bp fragment only in the male and hermaphrodite individuals (Fig. 3). This suggests that the specific DNA region of PSDM exists in the male and hermaphrodite, but not in the female, and the presence or absence of PSDM in our RAPD experiments was not caused by base changes at the primer-binding sites. Thus, SCARps converted from PSDM is an effective marker for the sex diagnosis of papaya seedlings.

Southern-blot analysis

Southern hybridization was carried out with genomic DNAs of the male line of IK-7, a female plant of cv

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1   TTGGCACGGG CTCTGAGCCA GGGTCGTGGT AAGAGTTTTT CCCAAGCCAT  50
    IBRC-RP07
51  TTTTTTCATT GCTTCTTCTC TTGAAATTTT AGTAAACTCA ATAAAACTTT 100

101 GGAAAAACTA ATTAAAATAA AAGGGCTAAT AATTCTACAT GAAAAATTTT 150

151 GCACGATTTA GATTAGATGT ATTGTAATTT TAAATTCGTT AAGTACCAAA 200
    ------------------------>SDP-1
201 AAGTTAGAGT ATTACAACAC TAAACTGGGC CGAACCTAGT TGGGGTCCAG 250

251 AGAATTAGGC AAGATCTCGC GGGGGTCAAT TAAACTGGAT TAGACCATCA 300

301 ACCTCTATAC AGACGTGCTA AGTAGCTGCA CAGCGCGACA CGCCATGGGA 350

351 GCAATGTGAC CTGGGCAAGC TATCCCGCAC GTGCACATCG CGCGTATATA 400
    SDP-2<------------------
401 AACCTGCGCA CTCAGCAAGA TAGCGCAGCA CAACATGCTG CCCGTGCCAA 450
                                                IBRC-RP07
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Fig. 2 DNA sequence of the papaya sex determination marker, PSDM. Annealing positions of the arbitrary primer IBRC-RP07 are *underlined*. *Arrows* indicate the positions of primers SDP-1 and -2, for amplifying the sequence-characterized amplified region marker, SCARps

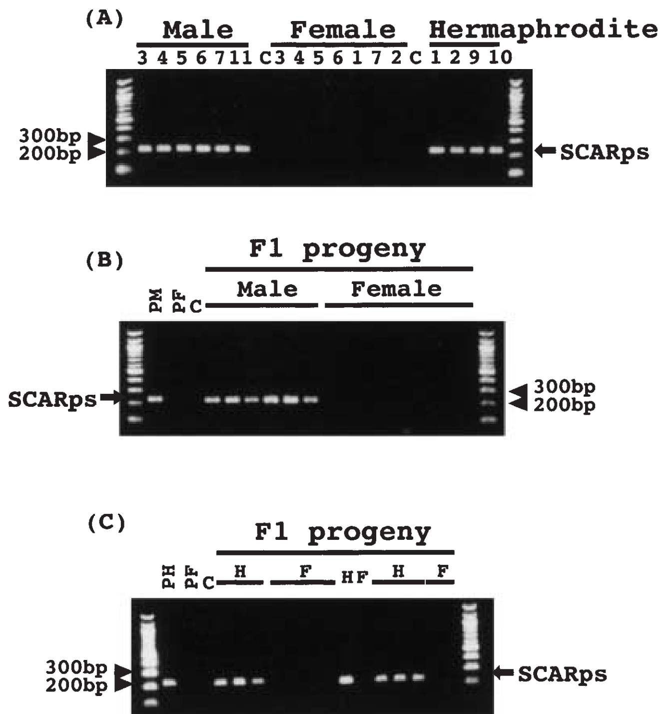

Fig. 3A–C The papaya SCAR marker discriminates sex. *C* no template. *SCARps* SCAR marker tightly linked to sex (fragments only in males and hermaphrodites). All samples analyzed were the same as in Fig.1

'Waimanalo Solo' and a hermaphrodite plant of cv 'Sunrise Solo', using a ^{32}P-labelled PSDM fragment as probe. Hybridization to *Eco*RI- and *Taq*I-digested DNAs showed bands in the male and hermaphrodite plants, but not in female plants (Fig. 4). This suggests that PSDM, occurring only in the male and hermaphrodite genomes, may be located in the chromosome region that is specific

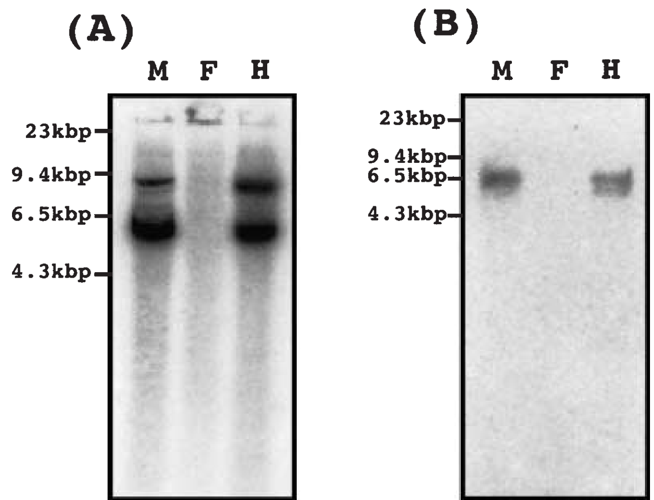

Fig. 4A,B Southern-blot analysis with ^{32}P-labelled PSDM. *M* male plant of IK-7; *F* female plant of cv 'Waimanalo Solo'; *H* hermaphrodite plant of cv 'Sunrise Solo'. (**A**) Southern-blot analysis of *Eco*RI-digested genomic DNA. (**B**) Southern-blot analysis of *Taq*I digested genomic DNA

to these sexes indicating the possibility of chromosomal differentiation between sexes. However, cytological studies have failed to observe heteromorphic sex chromosomes (Hofmeyr 1939). In Storey's (1953) zygotic lethality hypothesis for the sex determination of papaya, a single locus (*Sex1*) with three alleles (*M*, *H* and *f*) is proposed. Assuming that Storey's hypothesis is correct, the PSDM fragment seems to be localized on the chromosome segment including *Sex1-H* and *Sex1-M* alleles. We hypothesize that chromosomes containing *Sex1-H*, *Sex1-M* and *Sex1-f* alleles are differentiated from each other, although this differentiation is not cytologically detectable.

In this report we show, that a RAPD marker, PSDM, and a SCAR marker converted from PSDM, SCARps, can be used to determine the sex of papaya plants at an early developmental stage. Sex was correctly predicted with this marker in each of 49 papaya plants tested for sex. The application of the SCARps marker for sex identification of papaya at the seedling stage will enormously facilitate the cultivation and breeding of papaya by saving time, space and labor cost, otherwise required to grow plants of undesirable sexes.

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