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RESEARCH ARTICLE

Detection of sequence characterized amplified region (SCAR) markers linked to sex expression in *Carica papaya* L.

E. Niroshini[1], J.M.D.T. Everard[2], E.H. Karunanayake[1*] and T.L.S. Tirimanne[3]

[1] *Institute of Biochemistry, Molecular Biology and Biotechnology, University of Colombo, Colombo 03.*

[2] *Coconut Research Institute, Bandirippuwa Estate, Lunuwila.*

[3] *Department of Plant Science, Faculty of Science, University of Colombo,Colombo 03.*

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Abstract: *Carica papaya* L. exhibits monoecious and dioecious plants that usually take six months for phenotypic manifestation. Nursery culling aided by sex-specific DNA markers was envisaged to alleviate the unnecessary cost incurred by farmers for maintaining unproductive male plants that contribute to 40-50% of the population. The mechanism of sex determination in papaya has been described as a tri-allelic single gene system with alleles, M_1-dominant for maleness, M_2-dominant for hermaphrodism and *m*-recessive for femaleness with diploid zygotes; M_1M_1, M_2M_2 and M_1M_2 being inviable. Bulked DNA samples of male, female and hermaphrodite plants were amplified by Random Amplified Polymorphic DNA-Polymerase Chain Reaction (RAPD-PCR) using 100 random primers. Twenty of most promising of these were analyzed among individual sex types. Two sex-specific fragments, OPC09-1.7 and OPE03-0.4 were associated with maleness and hermaphrodism. The segregation of these two markers was analyzed in the F_1 population obtained by self-pollinating a hermaphrodite plant. A linkage was detected between the RAPD markers, OPC09-1.7 and OPE03-0.4 and the male and hermaphrodite sex of papaya plants. These sex-specific RAPD fragments were cloned and sequenced for converting them to more authentic SCAR markers. The Southern blot hybridization of RAPD-PCR products obtained by amplification of female, male and hermaphrodite papaya DNA amplified by OPC09 primer using radio labeled recombinant plasmid detected a polymorphic fragment in male and hermaphrodite papaya sex types. The nucleotide sequence of OPC09-1.7 fragment showed the possibility of developing more authentic SCAR markers to enhance the accurate sex determination of *Carica papaya* at the nursery stage.

Keywords: *Carica papaya,* papaya, SCAR marker, sex linked polymorphism

INTRODUCTION

Carica papaya L. (Papaya or Papaw), a native of tropical America, is a widely distributed fruit crop throughout the tropical and warmer subtropical regions. The consumption of papaya is growing steadily in parallel with the increase in health conscious food consumers, as the fruit is low in calories and sodium, but high in dietary fiber, calcium, potassium and vitamins A and C[1,2].

Papaya has attractive agronomic features such as easy cultivation, rapid growth, minimum growing space, early production, high yields, multiple uses, prompt returns, and adaptation to diverse (climatic and soil) growing environments[3]. Research on crop improvement conducted so far has shown immense opportunities such as diminished stature, uniform fruiting, increased fruit size and quality, fruiting precocity, and tolerance to various abiotic stress conditions (e.g. cold temperature)[4,5]. The *C. papaya* trees exhibit sexual polymorphism as female, male and hermaphrodite plants and discrimination can only be defined in 5 – 8 month old plants. One striking feature is the close association between sex expression and desirable agronomic traits. Hermaphrodite plants produce fleshy fruits of attractive shape, while female plants produce fruits rich in papain. Male plants are unproductive and growers incur high costs to maintain them for a substantial period of time. Identification of sex-specific DNA markers would assist in alleviating this problem, if such linked markers are reliable and unaltered by the environment.

*Corresponding author

Storey[6] suggests that sex phenotypes of papaya are conditioned by a tri-allelic single gene with alleles, M_1 (dominant for maleness), M_2 (dominant for hermaphrodism), and *m* (recessive for femaleness). The proposed model fitted well assuming that the diploid zygotes (M_1M_1, M_2M_2 *and* M_1M_2) are uniformly inviable. Parasnis *et al*.[1] provided molecular evidence for a putative Y chromosome in papaya that is associated with sex expression.

Previous studies have provided preliminary data indicating that Random Amplified Polymorphic DNA (RAPD) markers might be useful for detecting sex expression in papaya[7-12]. Moreover, papaya male and hermaphrodite sex linked RAPD and SCAR markers have been identified by Deputy *et al*.[13] and Urasaki *et al*.[11,12]. Genetic relationships among different *C. papaya* cultivars have been analyzed using amplified fragment length polymorphic (AFLP) markers[5]. Results indicated that the genetic variations of self-pollinated hermaphrodite cultivars were similar to the open-pollinated dioecious cultivars. Others had developed a high-density genetic map of papaya using 54 F_2 plants derived from cultivars Kapoho and SunUp with 1501 markers, including 1498 AFLP markers, the papaya ring spot virus coat protein marker, morphological sex type, and fruit flesh colour[14]. This map revealed severe suppression of recombination around the sex determination locus with a total of 225 markers co-segregating with sex types. A high level of DNA polymorphism in a genomic region surrounding the mapped sex locus named as male - specific region (MSY) has also been found[15]. This region was identified as it harbors male and hermaphrodite specific genes crucial for floral sex expression.

In this paper the detection of RAPD markers and SCAR markers with tight linkage to sex that could potentially be used to determine the sex of papaya seedlings is described.

METHODS AND MATERIALS

Thirty *C. papaya* plants, 10 from each sex type female, male and hermaphrodite (bearing flowers and fruits) were selected from home gardens around Kadawatha. The tender leaves were collected and sealed in polythene bags until they were used for DNA extraction. Total genomic DNA was isolated by using the simplified CTAB protocol[16]. DNA concentration was determined using the GeneQuant spectrophotometer (Amersham Pharmacia, Uppsala, Sweden). DNA amplification for RAPD analysis was carried out using 100 arbitrary decamer primers of the series OPA01-20, OPB01-20, OPC01-20, OPD01-20 and OPE01-20 (Operon Technologies, USA)[17]. Initial amplification was carried out using bulked DNA samples from each of the female, male and hermaphrodite plants separately.

Polymerase chain reactions (PCR) were performed in 25 µL volumes comprising 1x PCR buffer (Promega, Madison, USA), 1.8 mM $MgCl_2$ (Promega, Madison, USA), 0.16 mM each dNTPs (Amersham Pharmacia, Uppsala, Sweden), 10 pmol primer, 1 unit of *Taq* polymerase (Promega, Madison, USA) and 75 ng of template DNA. Amplification was performed using PTC-100 thermocycler (MJ Research, Inc., USA) with 45 cycles of 94°C for one min, 36°C for one min and 72°C for two min. Amplificons were separated on 1.2% agarose gel. Gels were scanned under UV by Photo-Print Gel Documentation system (Vilber Lourmat, France).

Polymerase chain reactions were repeated with 20 primers, based on their well-resolved amplification profiles and the presence of polymorphism among sex types. A hermaphrodite papaya plant with a green petiole was selected and used as a parent plant for self-pollination. DNA was extracted from 15 individuals[18] and RAPD markers among the individuals were analysed using OPC09 (CTCACCGTCC) and OPE03 (CCAGATGCAC).

The RAPD fragments, OPC09-1.7 and OPE03-0.4 found linked to male and hermaphrodite sex types were cloned: the DNA fragment was excised from an ethidium bromide stained 1% low melting point agarose gel and then purified using a GFX PCR DNA and Gel Band purification kit (Amersham Pharmacia, Uppsala, Sweden). The end filled PCR fragments (seven units of Klenow polymerase I, 50 mM Tris HCl pH 7.5, 10 mM $MgCl_2$ and 10 mM DTT, 2 mM each dNTPs, and 3 µg of eluted DNA), were purified and blunt ligated into *EcoR*V site of pBS vector and transformed into *E.coli* strain of XL1-BlueMRF[19].

DNA of female, male and hermaphrodite plants were analyzed using OPC09 RAPD. Amplicons were resolved on 1% agarose gels and blotted into Hybond N^+ membrane (Amersham Pharmacia, Uppsala, Sweden) with 0.5N NaOH transfer buffer according to Sambrook *et al*.[18]. The filters were hybridized with αP^{32} dCTP labeled plasmids harboring a OPC09-1.7 fragment. After stringency washing, the filters were exposed to X-ray film (Kodak XAR-5).

The inserted fragments of OPC09-1.7 and OPE03-0.4 were sequenced using Thermo sequenase Cy 5

Dye Terminater Kit according to the manufacturers' instructions (Amersham Pharmacia, Uppsala, Sweden) and ALFexpress automated sequencer (Amersham Pharmacia, Uppsala, Sweden). The sequences were analysed using GeneJockey (Biosoft, Cambridge, UK) computer software. Nucleotide sequence of OPC09-1.7 fragment of hermaphrodite papaya was submitted to NCBI database under the accession number AY063773. These sequences were used for a BLASTN search of NCBI databases.

Extended oligonucleotides or SCAR primers were designed for OPE03-0.4 and OPC09-1.7 fragments as E03/20FP (CCAGATGCACTTAGCAGGAAG) and E03/20RP (CCAGATGCACGGCGAATTTAGAGC) and C09/20FP (CTCACCGTCCATTTTAATTA) and C09/20RP (CTCACCGTCCGCGGCATCAATGTA), respectively.

These SCAR primers were used for the amplification of DNA obtained from female, male and hermaphrodite papaya plants. The PCR reactions were optimized: 1XPCR buffer 1.8 mM $MgCl_2$, 2.5 mM dNTPs, 10 pmol primer (forward and reverse) (Sigma Chemical Company, USA), 50 ng template DNA and 0.2 unit *Taq* polymerase in 25 µL reaction and the duration for denaturing (94°C for 30 s), annealing (58°C for 30 s) and extending (72°C for 120 s). The PCR proceeded for 30 cycles. The amplification products were resolved in 1.2% agarose gel.

RESULTS

Among 100 primers evaluated, 75 produced clear DNA profiles yielding a total of 971 amplification bands for the bulked sample of female, male and hermaphrodite papaya plants of which 89 bands were polymorphic. The best twenty primers upon screening of 12 individuals (four of each sex type) yielded 927 bands (strongly amplified) of which 357 were polymorphic.

Among all the polymorphic fragments only two polymorphic fragments amplified by OPC09 and OPE03 primers were found present in all the male and hermaphrodite types of papaya plants. Therefore, these two amplicons OPC09-1.7 kb and OPE03-0.4 kb (Figures 1 & 2) were considered as associated with maleness and hermaphrodism of papaya.

To observe the segregation pattern of these two RAPD markers OPC09 – 1.7 and OPE03 – 0.4, a family containing 14 individuals was obtained by self pollinating a hermaphrodite papaya plant (M_2m). This fruit contained only 17 seeds and out of that, only 14 seeds germinated and matured until flowering stage. According to the PCR analysis, two RAPD markers OPC09-1.7 and OPE03-0.4 showed a close linkage with the hermaphrodite and male sex types of papaya (Figures 3 & 4). Both markers gave an identical banding pattern with regard to segregation of two markers in the family, and the phenotypic expression of the individual members (hermaphrodism and femaleness) of the family agreed with the marker expression. Southern hybridization of female, male and hermaphrodite papaya with the labeled plasmid containing the insert OPC09-1.7 hybridized to 1.7 kb and 978 bp positions (Figure 5). The sequence of the insert was found to be 978 bp. Since this fragment contained OPC09 primer site at 5' and 3' ends and hybridized to 1.7 kb and 978 bp positions to produce polymorphic fragments, the SCAR primers designed to the fragment of 978 bp. Two SCAR primers, C09/20FP and C09/20RP amplified two fragments of length 1.7 kb and a 978 bp in both male and hermaphrodite plants confirming the male and hermaphrodite specific RAPD-PCR markers (Figure 6).

The sequence of polymorphic RAPD fragment of OPE03-0.4 contained 352 bp. No polymorphisms detected from female, male and hermaphrodite types of *C. papaya* from SCAR primers designed from this 352 bp fragment, E03/20FP (CCAGATGCACTTAGCAGGAAG) and E03/20RP (CCAGATGCACGGCGAATTTAGAGC).

BLASTN search of 978 bp and 352 bp fragments showed no compatibilities with the available *C.papaya* sequences in National Centre for Biotechnology Information (NCBI) databases.

DISCUSSION

The results illustrate the possibility of developing a molecular marker based method to identify sex at seedling stage in *C. papaya*. A considerable number of agriculturally important plants including nutmeg (*Myristica fragrans* Houtt.), hemp (*Cannabis sativa* L.), pistachio (*Pistacia vera* L.), kiwi fruit (*Actinidia chinensis* P.), asparagus (*Asparagus officinalis* L.) and papaya are dioecious. Farming of these crops could greatly benefit by development of methods for sex detection at an early stage as a sufficiently larger number of productive hermaphrodite or female (depending on the market preference) plants could be cultivated by minimizing the number of unproductive male trees.

The genetic mechanisms involved in dioecy vary in different plants. For papaya, there is evidence to suggest that male and hermaphrodite plants are heterogamous (XY) while female is homogamous (XX)[20]. According

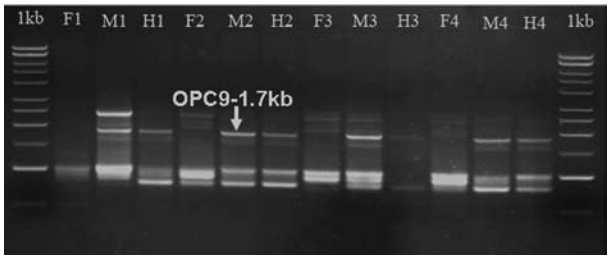

Figure 1: RAPD-PCR DNA profiles obtained by amplification of individual DNA samples of female (F), male (M) and hermaphrodite (H) plants of *C. papaya* L. using primer OPC09. Lane 1 and 14-1 kb ladder marker. The arrow ndicates 1.7 kb size fragment which is polymorphic to male and hermaphrodite sex types of papaya.

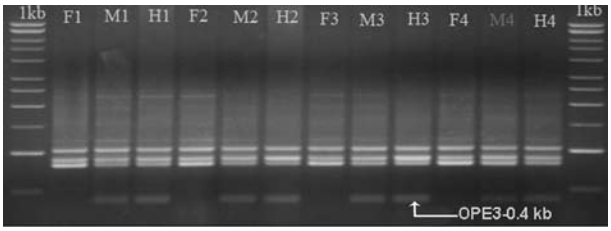

Figure 2: RAPD-PCR DNA profiles obtained by amplification of individual DNA samples of female (F), male (M) and hermaphrodite (H) plants of *C. papaya* L. using primer OPE03. Lane 1 and 14 -1 kb ladder marker. The arrow indicates 0.4 kb size fragment which is polymorphic to male and hermaphrodite sex types of papaya.

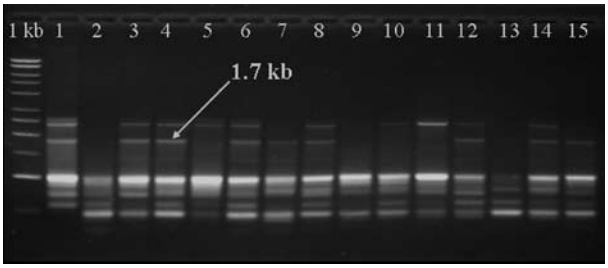

Figure 3: RAPD-PCR profiles of hermaphrodite parent and the F1 individuals amplified with primer OPC09. Lane 1-1 kb ladder marker, lane 2 - hermaphrodite parent (Hp), lanes 3 to 14 - F1 individuals. The arrow indicates 1.7 kb size polymorphic fragment present in all male and hermaphrodite sex types of papaya.

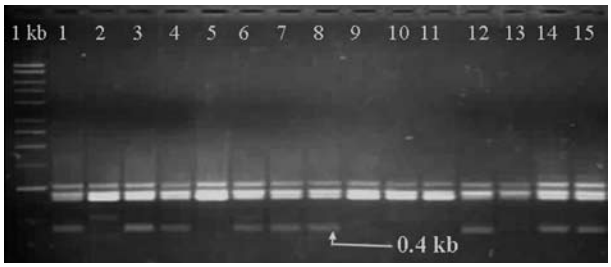

Figure 4: RAPD-PCR profiles of hermaphrodite parent and the F1 individuals amplified with primer OPE03. Lane 1-1 kb ladder marker, lane 2-hermaphrodite parent (Hp), lanes 3 to 14 - F1 individuals. The arrow indicates 0.4 kb size polymorphic fragment present in all male and hermaphrodite sex types of papaya.

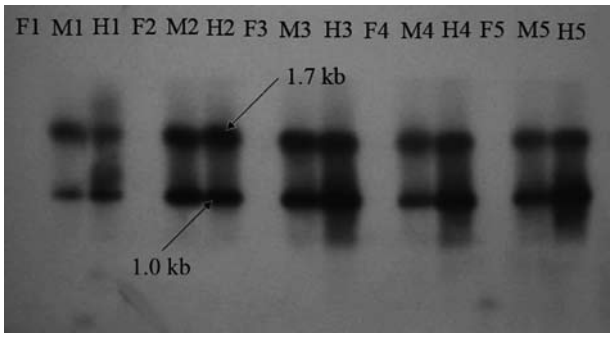

Figure 5: Autoradiogram of RAPD-PCR profile of female (F), male (M) and hermaphrodite (H) individual DNA amplified with primer OPC09 and hybridized to recombinant plasmid of OPC09-1.0 kb. Lanes 1 to 5 - individual DNA samples of particular type. The autoradiogram shows the two polymorphic fragments approximately 1.0 kb and 1.7 kb present in male and hermaphrodite papaya plants. The empty lane belongs to female plants and shows no signs of hybridization.

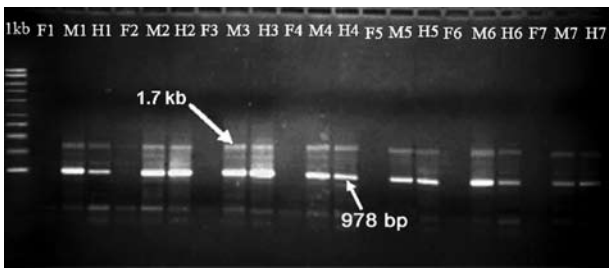

Figure 6: PCR profiles of female (F), male (M) and hermaphrodite (H) individuals of *C. papaya* L. DNA amplified with SCAR primer C09/20. Lane 1-1 kb ladder marker, lanes 2 to 22 - individual papaya DNA samples (F1-F7, M1-M7 and H1-H7). The arrows indicate two polymorphic fragments 978 bp and 1.7 kb present in all male and hermaphrodite sex types.

to others, the Y chromosome evolved in hermaphrodites and then converted to males by mutations[15]. As suggested by them, gynodioecy is an intermediate step to dioecy in the family Caricaceae. Dioecy has evolved to promote cross pollination in papaya.

Liu *et al*.[15] identified The male specific (MSY) region in hermaphrodite and male papaya plants had been identified and found that some sequences of this region are common in both male and hermaphrodite plants[15]. In our studies the fragments of 978 bp and 352 bp shared identical sequences between male and hermaphrodite type papaya, and this may support the above study.

According to the BLASTN search no sequence similarities were found between 978 bp and 352 bp fragments and the sequences of the MSY region.

The two polymorphic fragments, OPC09-1.7 and OPE03-0.4 appear to have the potential for development of a molecular marker based technique for early identification of papaya sex expression. Further, the observations obtained from the segregation of two RAPD-PCR fragments OPC09-1.7 and OPE03-0.4 agreed with a simple Mendelian type supporting the hypothesis described by Hofmeyr[20] that males and hermaphrodites are heterozygous. The fragments OPC09-1.7 and OPE03-0.4 produced clear polymorphisms in DNA samples of heterozygous parent and individuals of F_1 progeny. According to Hofmeyr[20] the hermaphrodite individual has the heterozygous allele M_2m and in the self-cross, the gamete containing either M_2 or m will fuse to produce zygotes M_2M_2, M_2m and mm. Since M_2M_2 is inviable, only M_2m (hermaphrodites) and mm (females) remain in the progeny. Since the gametes carrying the M_1 allele are not produced in the hermaphrodite parent, males would not arise from self-mating, and the population actually consisted only of 10 hermaphrodites and six females giving an approximate ratio of two hermaphrodites to one female, confirming the phenotypic ratio of 2:1 of a self-crossed family.

The SCAR marker derived from OPC09-1.7 showed sex specificity to male and hermaphrodite plants which were confirmed by Southern hybridization. Therefore this fragment has the potential to be developed as a sex specific molecular marker.

Although primer OPE03 detected a sex specific polymorphism in the RAPD-PCR analysis, the SCAR primers designed failed to detect the sex specific polymorphism. The polymorphism detected by OPE03 primer in the RAPD-PCR analysis may be due to a single base differences or single base mutation at the primer annealing site. Since SCAR primers contain ten more bases at 3' end, SCAR amplification failed to detect this single base polymorphism. To design a primer that can detect this single base difference and to design a primer that can detect sex specific polymorphism, it is important to design "allele" specific primers to female DNA after cloning and sequencing of the fragment containing 352 bp produced by the SCAR primers.

Detection of sex-linked RAPD markers as well as the SCAR markers have been attempted in several dioecious species. It has been identified that 32 male-specific RAPD bands in hop, (*Humulus lupulus* L.) by screening 900 random primers[21] and others found one RAPD fragment of 400 bp size, closely linked with male sex type of hemp (*C. sativa* L.)[22]. Pointed gourd (*Trichosanthes dioica* Roxb.) has also been studied and found to have a RAPD marker associated with females, that is absent in all male plants[23]. Similarly, presence of a female-specific band in nutmeg, (*M. fragrans* Houtt.) has also been reports by screening 60 Operon primers[24]. Others have detected two RAPD markers linked to *M* locus (maleness) in Asparagus (*A. officinalis* L.) and successfully converted one of these bands to a SCAR marker[25].

According to this study it is suggested that the SCAR marker OPC09-1.7 and the RAPD markers OPC09-1.7 and OPE03-0.4 can be used for developing a single PCR diagnostic assay for sex determination in *C. papaya* L.

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