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Development of sex-linked PCR markers for gender identification in papaya

Chaithra T.S., Kanhaiya Singh, Jai Prakash*, S.K. Singh, Zakir Hussain, N.C. Gupta and R.R. Kumar

Division of Fruits and Horticultural Technology, ICAR-Indian Agricultural Research Institute, New Delhi 110012, Delhi, India

ABSTRACT

Papaya is an important fruit crop in the tropical and subtropical regions of the world, having several nutritional and numerous medicinal properties. It is a polygamous plant that has three primary sex forms, *viz*., male, female and hermaphrodite. Several methods are employed, based on morphological, physiological, and cytological factors, to detect the sex of plants at an early stage of growth. However, none of them proved effective, which led to the development of highly effective gene-based markers. In the present study, attempts were made to validate the molecular markers to determine the sex during the early stage of the plant. Out of the ten selected RAPD-SCAR markers, five SCAR markers showed amplification. Among the amplified markers, SDP, PKBT 5, and PMSM 2 proved most effective for identifying sex at the early developmental stage. These markers can be used commercially to select desired seedlings at an early stage for plantation.

Key words: *Carica papaya* L., Juvenile stage, Sex identification, RAPD-SCAR markers.

INTRODUCTION

Papaya (*Carica papaya* L.), native to Central and South America, is an important fruit crop in tropical and subtropical regions of the world. The cultivated papaya is closely related to *Vasconcellea* sp., which shared a common ancestor with *Arabidopsis thaliana*, a member of the order Brassicales (Ming *et al*., 13). Papaya belongs to a polygamous flowering plant, which has three primary sex forms: male, female, and hermaphrodite (Storey, 16). Female and hermaphrodite flowers are on short cymes, while male flowers are on a highly elongated cyme panicle (Storey, 17). Sex determination has drawn the attention of the breeders due to its exciting biology and economic consequence caused by the segregation of papaya sex. There are several hypotheses for the sex determination of papaya that have been independently proposed over the years by different scientists. Based on the segregation ratio among different sex types, Hofmeyr (7) and Storey (16) separately proposed that the sex expression in papaya is controlled by a single gene with alleles, namely 'M1' being dominant allele for male, 'M2' another dominant allele for hermaphrodite and 'm' a recessive allele for female sex type given by Hofmeyr, while Storey gave 'M^h' for hermaphrodite allele, 'M' for male individual and 'm' for female. Of this dominant combination of MM, M^hM^h and MM^h are lethal, while M^hm and Mm genotypes are viable due to active sex chromosomes. Hofmeyr (7) proposed the genic balance hypothesis, which states that factors that determine femaleness are present in the sex chromosome and that of maleness in the autosome. Later, in 1953, Storey reported that the papaya sex is not determined by a single gene but by a group of genes, which are closely linked and present on the sex chromosomes. Sondur *et al*. (15) gave the concept of sex determination and proposed that *SEX1-M* is responsible for the development of stamens, thereby regulating the male sex type, while *SEX1-H* which regulates hermaphrodite sex and *sex-f*, gives rise to female sex type (Leela *et al*., 8). Liu *et al*. (11) proposed a hypothesis that chromosome pair XX and XY control sex determination in papaya; XX controls the female sex, and XY controls males. The male-specific region tends to occupy 10% of the Y chromosome and 13% of the Yh chromosome of hermaphrodites (Zhang *et al*., 20). Papaya is an excellent model plant for sex determination and other genomic studies because it has a small genome size (372 Mbp), short generation time (9 months), and wider genetic variability due to propagation by seeds (Liu *et al*., 11).

Propagation of papaya through seeds is still a commercial method due to the absence of commercial vegetative means of propagation. However, propagation by seeds of dioecious varieties will produce male and female plants, and identification of the sexes of these plants at the juvenile stage is difficult as they look morphologically similar. The sex of these plants can be determined only after 6-8 months of planting at the reproductive stage; hence, 2-3 plants in a single planting site are recommended.

*Corresponding author: singhjai2001@rediffmail.com

So, maintaining an appropriate sex ratio of female and male plants is problematic. Therefore, sex identification at a seedling stage before transplanting has immense advantages in papaya, as it avoids planting excess undesirable plants, and it will also save the efforts, inputs, and cost of cultivation.

Prediction of the sex of the plants at earlier stages prior to planting has been tested by several workers using morphological, cytological, biochemical and PCR-based gender-linked markers. Morphological markers include the colour of the seed coat, leaf and root morphology, petiole length, thickness and orientation and seedling stem colour. In this case, the male has a dark seed coat colour and a straight root shape (Magdalita and Mercado, 12); along with this, Elliptic Fourier Analysis (EFA) was developed by Demandante *et al*. (3) for sex identification. Cytological identification, which includes the identification of chromatin bodies or the existence of heteromorphic chromosomes, helps in sex determination in papaya (Datta, 2). Apart from this, some biochemical markers that include phenolic content and isozymes, which differ in the amino acid sequence, play a vital role in the sex identification of papaya.

Nevertheless, these enzymes have several disadvantages, and mainly, they are affected by the environment and stage of the plant, and the expression of these enzymes varies from tissue to tissue. Hence, none of these methods are consistent for the prediction of sex at the seedling stage. It led to the Utilization of DNA-based molecular markers using PCR technology as a reliable tool for determining the sex in papaya. Nowadays, RAPD-SCAR markers have been utilized successfully for sex determination in papaya (Deputy *et al*., 4). Utilizing such identified markers along with some new markers for validation in popular papaya cultivars may be very effective.

MATERIALS AND METHODS

The experiment was conducted during 2020-21 in the experimental orchard of the Division of Fruits and Horticultural Technology, ICAR-IARI, New Delhi, in a factorial randomized Block Design with three replications. The experimental materials comprised of six divergent papaya genotypes, namely, Red Lady (RL), P 9-5, P 7-2 (gynodioecious) and Pune Selection 3 (PS 3), Pusa Nanha (PN), Pusa Dwarf (PD) (dioecious). As the cultivated papaya has a polygamous flowering habit, the selection of both gynodioecious and dioecious represents all the sex forms governed by different genes. The sex of selected plants of all six genotypes was assessed using RAPD- SCAR markers at the early growing stage using 10 primers. Genomic DNA from the leaves of papaya plants was extracted using the CTAB method (Doyle and Doyle, 5) with slight modifications. Qualitative assessment of DNA was done by using agarose gel electrophoresis. Isolated genomic DNA was electrophoresed on 0.8% agarose gel, which was stained with ethidium bromide, and Hind III-cut λ DNA was taken as a standard and run at 80V and 70mA for two hours in 1X TAE Buffer and then observed on UV- trans-illuminator (CLiN). The quality of DNA was judged by whether the DNA sample formed a single high molecular weight band or smeared. Nanodrop assessed the quantity of DNA. The concentration of DNA was calculated based on the absorbance at 260 nm using the following formula:

$$\text{DNA (µg/ml)} = \frac{\text{OD260} \times \text{dilution factor} \times 50}{1000}$$

The concentration of DNA can be detected by the ratio of absorbance at 260 nm and at 280 nm.

Markers analysis with a working concentration of 50 ng/µl DNA was prepared by diluting the DNA for further PCR reaction. SCAR and RAPD markers working solution was prepared. 10 µl of the reaction mixture was prepared by adding 1 µl of 50 ng/µl template DNA, 1 µl of forward and 1 µl of reverse primers, and 5 µl master mix (Gene Dire X, Inc.) and the final volume was made by adding 2 µl nuclease-free water. All the primers were amplified using PCR (Prima-96 plus). SCAR and RAPD analysis was conducted by utilizing published markers 71E, SCO08, PVF 1 & PVF 2 (Yakubov *et al*., 19), SDP (Urasaki *et al*., 18), PKBT 4 & PKBT 5 (Sobir and Pandia, 14), SMY 1(Atak *et al*., 1), W11 (Deputy *et al*., 2002), SPD (Urasaki *et al*., 2002) and a new SCAR marker PMSM 2 (Liao *et al*., 10). For the SCAR marker, PCR reactions were undertaken under the following conditions: Amplification as pre-incubation period at 94°C for 4 min., followed by 35 cycles of denaturation at 94°C for 1 min., annealing at 54-58°C and extension at 72°C for 1 min. and final extension of 72°C for 7 min. For the RAPD marker, PCR reactions were undertaken under the following conditions: The initial cycle was at 94°C for 3 min., followed by 45 cycles at 94°C for 1 min., 34°C for 1 min., 72°C for 2 min., and a final extension at 72°C for 5 min.

RESULTS AND DISCUSSION

A total of 10 SCAR and RAPD markers, namely, 71E, SDP, SMY 1, PKBT4, PKBT5, PMSM2, OPO-08, SCO-08, PVF 1, PVR 2 and SMY 1 were used in 48 papaya plants of 6 genotypes, namely, 3 gynodioecious (Red Lady, P-9-5 &P-7-2) and 3 dioecious (Pune Selection 3, Pusa Nanha and Pusa Dwarf) for the sex identification at early stage. Among 10 SCAR-RAPD markers, only four markers, *viz*., PKBT4, PKBT5, SDP, and PMSM2, were amplified

for that sex locus, and another marker 71E yielded the amplification in all the 48 tested samples (Fig. 1). The other five markers failed to amplify any of the six genotypes tested. The sequence information of the primers is presented in Table 1.

Among amplified SCAR markers, PKBT5, SDP and PMSM2 showed higher levels of accuracy. Another marker, 71E (Fig. 1), amplified all three sex types, namely male, female and hermaphrodite, and this marker can be taken as a control. This marker produced amplicons for all three sex types at 1000 bp because the sequence was present on autosomes, but they were not polymorphic. A similar result was also reported by the study of Liao *et al*. (10). Another SCAR marker, SDP (Fig. 2), amplified a fragment length of ~ 220 bp, indicating the presence of the *SEX1* gene. In this marker, the desired bands were present in male and hermaphrodite plants. However, no bands were obtained for female plants in all the dioecious and gynodioecious genotypes studied. This is one of the most effective markers for identifying male and hermaphrodite plants in papaya at the early seedling stage. Urasaki *et al*. (18) and Leela *et al*. (8) also reported similar results with this marker. They found that this marker is tightly linked with the

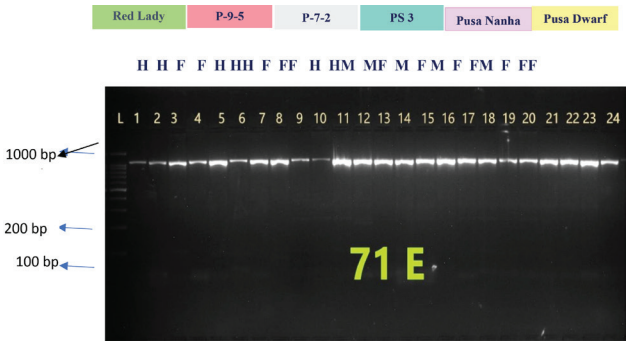

Fig. 1. PCR amplification for the marker 71 E for all three sex types of papaya of six genotypes. Names of all the genotypes were depicted in the colour boxes, L- 100 bp Ladder, H- hermaphrodite, M- male, F-female and the numerical numbers on the gel image, which represents the samples that are used in the experiment.

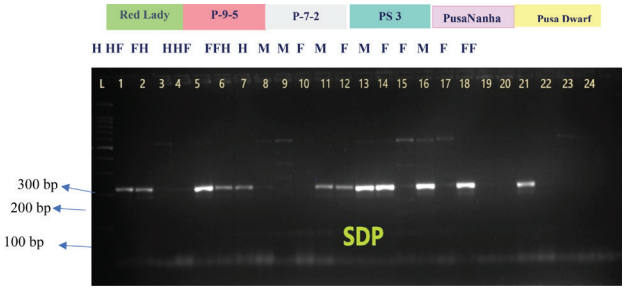

Fig. 2. PCR amplification for the SCAR marker SDP for all papaya sex types. Genotypes names were depicting in the colour boxes, H- Hermaphrodite, F-female, L- 100bp ladder and numerical on gel image represents the samples number which were utilized in the experiment.

Table 1. List of primers and their annealing temperature, which were used to identify the sex of the papaya plants in the early growing stage.

Primer	Primer sequence	Reference	Annealing temperature	Band size
71E	F: TTCCATCGAGCTTCCTAGTC R: GCGACTACTCTACCATGCTT	Yakubov *et al.* (19)	57°C	1000 bp
SDP	F: GCAGGATTTAGATTAGATGT (20) R: GGATAGCTTGCCCAGGTCAC (20)	Urasaki *et al.* (18)	54°C	~ 250 bp
PKBT4	F: GAGGGCGAGGTTTGAATTTGG (21) R: TTTGGTGTCCTGGTTACCCTC (21)	Sobir and Pandia (14)	56°C	~ 320 bp
PKBT5	F: AGCCAGGGTCGTGGTAAGAG (20) R: TCCCATGGCGTGTCGCGCTG (20)	Sobir and Pandia (14)	55°C	~ 350 bp
PMSM2	F: GCGATGCTTCAAGTGTTGAC R: ACTATGAGCCTCACGCACTA	Liao *et al.* (10)	55°C	500 bp
OPO-08	CCTCCAGTGT	Yakubov *et al.* (19)	Not amplified	-
SCO-08	F: CCTCCAGTGTGAATCAAGTAAAC R: CCTCCAGTGTTATGTAATACCAAAA	Yakubov *et al.* (19)	Not amplified	-
PVF1	GTCGTAGATGAAAACACC	Yakubov *et al.* (19)	Not amplified	-
PVF2	TAATAGAAGCCATAGA	Yakubov *et al.* (19)	Not amplified	-
SMY 1	F: TCGCAATTCGTTAGGGATGATGCG R: CATAATCAACCATCCATAAAAACCAT	Atak *et al.* (1)	Not amplified	-

SEX1 gene, which determines the sex expression in papaya. Liu *et al*. (11) also proposed that the MSY region is the probable location of this sequence where the genome of male and hermaphrodite sex type shares close identical DNA sequences. The marker PKBT 4 (Fig. 3) amplified the male and hermaphrodite plants at ~350 bp. This result did not confirm the findings of Lemos *et al*. (9), who detected bands at fragment size 438 bp. It showed that this marker is not consistent in determining the sex of papaya from different genetic backgrounds. Sobir *et al*. (14) also reported similar results. Among the SCAR markers that produced sex polymorphism, PKBT 5 (Fig. 4) showed 100% reliability in the sex identification of papaya. This marker amplified at ~ 350 bp for male and hermaphrodite plants with no bands for female plants. This marker could effectively differentiate male and hermaphrodite from the female papaya plants in the early pre-bearing stage from the different genetic backgrounds, as had also been reported by Lemos *et al*. (9), Sobir *et al*. (14) and Ejaz *et al.* (6). A robust male-specific marker PMSM 2 (Fig. 5) (Papaya male-specific marker 2) was precisely designed to detect the male-specific regions in papaya genotypes. In the RAPD-SCAR marker, PMSM 2 amplification occurred at ~500 bp in males only, which may be due to the probable location of this marker on the male-specific region of the Y chromosome. This male-specific marker was developed using sequence information of HSY, MSY, and X. MSY and HSY share 99.6% of sequence identity; therefore, the chances of amplification of male and hermaphrodite plants are higher as compared to females. This marker can differentiate the dioecious and gynodioecious cultivars of papaya. A similar finding was also reported by Liao *et al*. (10) in papaya. Other selected markers, PVF 1 and PVF 2, and another SCAR marker, SCO 08,

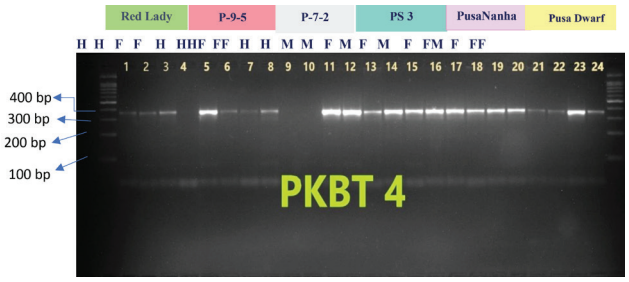

Fig. 3. PCR amplification representing the segregation of SCAR marker PKBT 4 with all three papaya sexes. Names of the genotypes are denoted in the colour boxes, H- Hermaphrodite, F-female, L- 100 bp ladder and numerical on gel image represents the samples number which were utilized in the experiment.

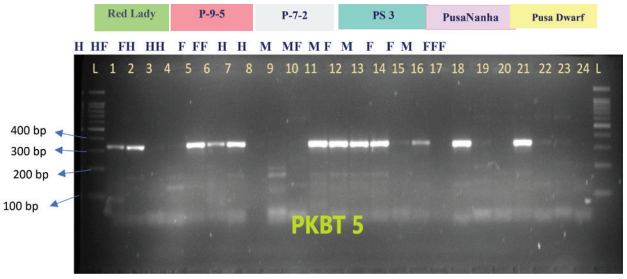

Fig. 4. PCR amplification representing the segregation of SCAR marker PKBT 5 with all three papaya sex types. Names of the genotypes are denoted in the colour boxes, H- Hermaphrodite, F-female, L- 100 bp ladder and numerical on gel image represents the samples number which were utilized in the experiment.

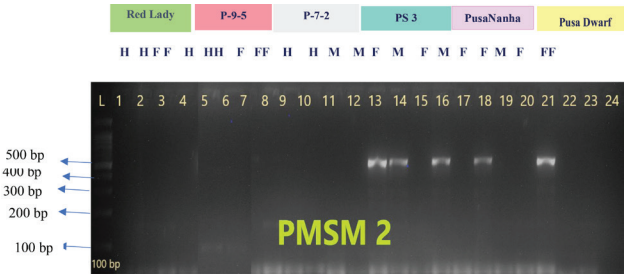

Fig. 5. PCR amplification representing the segregation of SCAR marker PMSM 2 with all three papaya sex types. Names of the genotypes are denoted in the colour boxes, H- Hermaphrodite, F-female, L- 100 bp ladder and numerical on gel image represents the samples number which were utilized in the experiment.

gave effective results in the case of pistachio nut, particularly in the identification of female plants (Yakubov *et al*., 19). In the case of papaya, they did not show the amplification in any samples tested due to the absence of a complementary sequence in the papaya sex chromosome. Another marker, SMY 1, which gave effective results as in the case of Atak *et al*. (1) in Kiwi fruit in the identification of sex at an early stage of growth, did not show any amplification in the case of papaya. The reliability of markers was known by observing the plants after flowering in the main field. Among the selected 48 samples of six genotypes, results obtained through marker were depicted in Table 2. The sample that showed amplification was denoted as '+', and the sample that did not show amplification was denoted as '-'.

The ideal sex identification method should produce consistent results and be relatively easy. When a marker for only one of the sexes has been identified, there is a risk that analysis results were false. The absence of a PCR product in the sample

Table 2. Showing sex confirmation by SCAR markers at an early developmental stage. Presence of amplified band denoted as '+' and absence denoted as '-'.

Sl. No.	Genotype	Sex prediction	SDP	PKBT 4	PKBT 5	PMSM 2
1.	Red Lady	H	+	+	+	-
2.		H	+	+	+	-
3.		F	-	-	-	-
4.		F	-	-	-	-
5.		H	+	+	+	-
6.		H	+	+	+	-
7.		H	+	+	+	-
8.		F	-	-	-	-
9.	P 9-5	F	-	-	-	-
10.		F	-	-	-	-
11.		H	+	+	+	-
12.		H	+	+	+	-
13.		H	+	+	+	-
14.		F	-	+	-	-
15.		F	-	+	-	-
16.		H	+	+	+	-
17.	P 7-2	F	-	+	-	-
18.		H	+	+	+	-
19.		F	-	+	-	-
20.		F	-	-	-	-
21.		H	+	+	+	-
22.		H	+	-	+	-
23.		F	-	-	-	-
24.		H	+	+	+	-
25.	Pune Selection 3	F	-	-	-	-
26.		M	+	+	+	+
27.		F	-	-	-	-
28.		M	+	+	+	+
29.		M	+	+	+	+
30.		F	-	-	-	-
31.		M	+	+	+	+
32.	Pusa Nanha	F	-	-	-	-
33.		M	+	+	+	+
34.		M	+	+	+	+
35.		F	-	-	-	-
36.		F	-	-	-	-
37.		M	+	+	+	+
38.		M	+	+	+	+
39.		F	-	+	-	-
40.	Pusa Dwarf	M	+	+	+	+
41.		F	-	+	-	-
42.		M	+	+	+	+
43.		F	-	+	-	-
44.		F	-	-	-	-
45.		M	+	+	+	+
46.		F	-	-	-	-
47.		M	+	-	+	+
48.		F	+	+	-	-

could be a negative result, but it could also elucidate the absence of DNA or other inconsistencies in the PCR process. A researcher should ideally utilize one or two distinct SCAR markers that produce products of different lengths in males and females during the same amplification process.

In the early stages of plant growth and development, concerted efforts are still needed to implicate potential sex-specific or sex-linked markers. Furthermore, these methods might be used to identify candidate genes that determine or influence the sex type. However, the interactions between genes and the environment may indicate a more complex mechanism. Research on sex-specific evolution and sex determination in the future should adopt a global perspective, including combining multiple levels of morphology, cytology, physiology, and molecular biology with multi-omics integration, such as whole genome sequencing, RNA sequencing, and proteomics. In addition, a few other high throughput molecular techniques that have yet to receive much attention, such as representational differential analysis (RDA), single nucleotide polymorphism (SNP) arrays and differential display analysis (DDA), have also proved effective.

AUTHORS' CONTRIBUTION

Conceptualization of research (CTS, JP, KS, SKS); Designing of experiments (CTS, KS, JP); Contribution of experimental material (JP, KS); Execution of field/ lab experiments and data collection (SKS, ZH, NCG, RRK); Analysis of data and interpretation (JP, KS); Preparation of manuscript (CTS, JP, KS).

DECLARATION

The authors declare that they do not have any conflict of interest.

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