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Sex determination of papaya plants derived from seed and micro-propagation

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ABSTRACT

Papaya has attained the status of 'Super food' with its inclusion in the daily dietary basket of people all around owing to its nutraceutical value. Being a polygamous species, determination of sex types in papaya proves to be a real challenging task. The aid of molecular techniques has been proved to be quick and reliable in identifying the sex types in plantlets growing in seedbed itself, which can save resources and time. The present study employed sex-linked DNA markers (sequence characterized amplified region or SCAR) to validate sex types in cv. Arka Surya and Inter-generic hybrid (IGH) seedlings and micro-propagated plants of papaya before transplanting in the intended field. These markers were checked in gynodioecious genotypes, and the results manifested clear segregation of females from hermaphrodites. Findings derived from three years of in vitro maintained mother cultures were the same as those of mature papaya plants. In conclusion, the markers T12 and W11 were the potential markers to identify the sex of papaya at the seedling stage. The SCAR used in this study has shown the great implication in selecting sex forms of papaya for tissue-cultured plants.

Keywords: *Carica papaya* L., SCAR marker, Gynodioecious, Sex types

INTRODUCTION

Papaya (*Carica papaya* L.), an important fruit crop with very high productivity has established its acceptability of commercial papaya cultivation in tropical as well as subtropical zones of India. It is best natural reservoir of vitamin A and C and is fairly rich in minerals (Sies and Stahl, 9). Papain manufactured from the dried latex of immature fruits, whose proteolytic action is similar to that of pepsin and trypsin, is used in the food, as well as in the textile, pharmaceutical, and cosmetic industries (Su *et al*., 12). It is basically polygamous in nature with three different sex forms *viz*., staminate, pistillate and hermaphrodite (Dinesh *et al*., 5).

The commercial mode of propagation for papaya is through seeds. Being cross pollinated nature, seed propagation gives rise to considerable range of variation. Commercial varieties, are typically either dioecious (staminate and pistillate flowers on different plants) or gynodioecious with female and hermaphrodite plants in the population. In gynodioecious varieties the fruits from bisexual flowers are more preferred as they have more economic value. Sometimes two seedlings /pit are planted, and when they start flowering phase, only hermaphrodites are retained (Wu *et al*., 14). Conventional vegetative propagation techniques such as layering, grafting and rooting of cuttings (Soomark and Tai, 11) have not resulted in efficient mass multiplication of hermaphrodite papaya. Another limitation is the segregation of staminate and pistillate types owing to the general dioecious nature of this crop. In this respect, tissue culture of selected hermaphrodite plants true to type in nature provide great benefit to the growers. Micro-propagation using shoot-tips from glass house grown plants or the use of shoot-tip and axillary buds from the lateral shoots of field-grown trees has been demonstrated in papaya (Wu *et al*., 14). In addition, for tissue culture, the early perception of the sex type of a particular papaya seedling would be beneficial, since the desired sex type can be selected prior to tissue culture. This will ensure that the resulting tissue cultured plants are 100% either females or hermaphrodites (Magdalita and Mercado, 7).

Molecular markers have been largely exploited for determination of sex type in papaya. A PCR-based method for quick and accurate determination of the sex of papaya was invented utilizing either W11 or T12 markers to determine the hermaphrodite or male allele or T1 marker, which amplifies a product regardless of sex type, as a positive control (Deputy *et al*., 4). Sex determination was done for both seedling and micro-propagated plants before transplanting in the major field by using T1 and T12 markers. Both markers produced amplified bands for hermaphrodite plants at 1300 and 800bp and were identified by the 800bp band that was lacking in the females (Fitch *et al*., 6). Assessment of the field performance of hermaphrodite plants

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by the conventional multi-planting practice to those resulting from marker-assisted selection (MAS) and micropropagation (Araya-Valverde *et al*.,1). Very few study have been made on field validations of sex of papaya plants selected by molecular marker with micro-propagaton. In this experiment, MAS has been used for determination of sex of papaya derived from tissue culture and seedling origin.

MATERIALS AND METHODS

A total of 92 papaya plants were used to test DNA markers which were of gynodioecious origin. The details of plant material used in this study are furnished in Table 1. It consisted of 64 Intergeneric Hybrids (IGH) (*Carica papaya* cv. Surya × *Vasconcellea cauliflora*) and 28 plants of Arka Surya. Their DNA were extracted before planting in the experimental plot. The investigation was organized at ICAR- Indian Institute of Horticultural Research, Bengaluru, Karnataka during 2017-2020. Genomic DNA was released from 100 mg of healthy leaf tissue utilizing standard CTAB technique with minor modifications (Saghai-Maroof *et al*., 8). DNA quantity was examined by a NANODROP 2000c, and also determined by an agarose-gel electrophoresis.

For seedling plants, seeds were taken for sowing from hermaphrodite selfed fruits and sown in polybags after treating with 100 ppm GA_3 for overnight. Seedlings were selected and labeled properly after 20 days and their sex was identified at seedling stage using molecular markers. Total of 50 plants (25 Arka Surya + 25 IGH) were transplanted in the field after 45 days and the observations with respect to flowering were recorded to confirm the sex type of the identified seedlings.

In case of micro propagated plants, flowers were selfed on the identified plants and seeds were taken out from the ripe fruits. Seeds were sown in the portrays after treating with 100 ppm GA_3. The shoot tips from the 14-15 days old seedlings were used as explants for *in vitro* multiplication. Cultures were initiated from shoot tips and identification of sex as carried out from the established cultures through MAS. In Arka Surya only three hermaphrodite cultures were maintained and 25 plants were generated from these cultures. Cultures were maintained separately to avoid accidental mixing up of both hermaphrodite and female seedling plants. In Intergeneric hybrids, a total of 39 mother cultures were initiated and labeled as IGH.SD.1 to IGH.SD.39. Three plants from each IGH mother cultures were generated totally to 117 plants. Hence a total of 192 plants consist of 25 plants of Arka Surya seedling origin, 25 plants of Arka Surya micro propagated, 25 plants of IGH seedling origin and 117 plants of IGH micro propagated were field planted and evaluated for sex determination.

PCR amplification of the SCAR (sequence-characterized amplified region) marker was carried out in 25-µl volumes having 10 ng of genomic DNA, 0.1 µM each of primers T1 (5´-TGCTCTTGATATGCTCTCTG-3´); T12 (5´-GGGTGTGTAGGCACTCTCCTT-3´) and W11 (5´-CTGATGCGTGTGTGGCTCTA-3´), 0.1 U of Ex Taq DNA polymerase (GeNei), 0.2 mM of dNTPs, 1.5 mM 10X *Taq* Buffer (including 20 mM $MgCl_2$). Amplification was performed for 30 cycles at 95°C for 5 minutes, 58°C for 1 min and 72°C for 1 min, followed by a final extension at 72°C for 7 min. PCR products were separated by electrophoresis in 1.0% agarose gels, and visualized by ethidium bromide staining. Touch down of 0.5°C for the first 20 cycles was carried out. Number of polymorphic bands generated by each primer was scored as absence or presence of band. Three primers (SCAR) were employed to distinguish male, female and hermaphrodite sex forms in seedlings and well differentiated adult plant types (Deputy *et al*., 4). Details of the primers used in the study are given in Table 2.

RESULTS AND DISCUSSION

SCAR T1, T12 and W11 primers were employed for the sex determination of all the plants addressed in this study. Since T12 and W11 primers only identify hermaphrodite genotypes, the reactions without amplification are regarded as females, which may however induce an error due to the DNA quality. T1

Table 1. Details of plant materials used and their source

S. No.	Genotype	No. of plants	Source
1	Arka Surya seedling plants	25	ICAR-IIHR, Bengaluru
2	IGH seedling plants	25	ICAR-IIHR, Bengaluru
3	Arka Surya micropropagated plants	3	ICAR-IIHR, Bengaluru
4	IGH micropropagated plants	39	ICAR-IIHR, Bengaluru

Table 2. Details of primers (SCAR) used in the study

Primer ID	Primer sequence (5'……..3')	Size of PCR product (bp)
T1	T1F:TGCTCTTGATATGCTCTCTG T1R:TACCTTCGCTCACCTCTGCA	1300
T12	T12F:GGGTGTGTAGGCACTCTCCTT T12R:GGGTGTGTAGCATGCATGATA	800
W11	W11F:CTGATGCGTGTGTGGCTCTA W11R:CTGATGCGTGATCATCTACT	800

SCAR primer, which was utilized as positive control to ensure that the reactions were successful, produced a 1,300bp product in all papaya specimen tested. T12 and W11 SCAR primers amplified a product approximately 800bp in hermaphrodites, but no amplicon was obtained in female plants.

SCAR W11 had the band size of ~800bp expressed only in hermaphrodite plants but not in female plants for papaya genotypes Arka Surya seedlings (25) (Figure 1), IGH Seedlings (25), IGH (22) different cultures and Surya (3) different cultures (Figure 2).Tests on two different planting materials (seedlings and micro propagated) of papaya indicate that a success rate for identification of hermaphrodite plants of over 100% can be obtained using this system. The results of the prediction by PCR were consistent with the observed sex type of the plants tested. The findings of SCAR W11 are in accordance with those of Deputy *et al.* (4) they reported same result after screening 182 F_2 population derived from

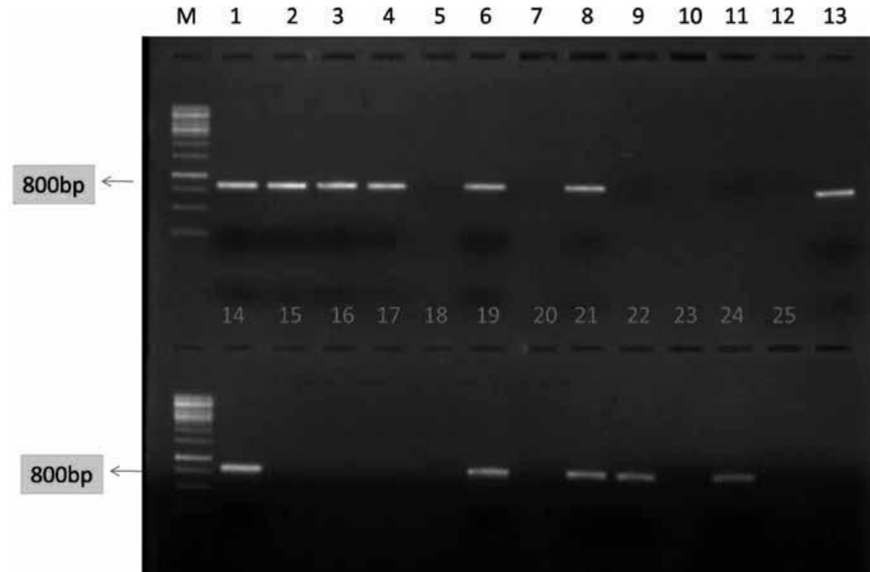

Fig. 1. PCR amplification indicating segregation of SCAR W11 with papaya sex.W11 SCAR marker giving an amplicon of 800bp size only in hermaphrodite, but not in female plants; M – 1 kb ladder; from 1 to 25: female and hermaphrodite papaya cultivar of Arka Surya seedlings.

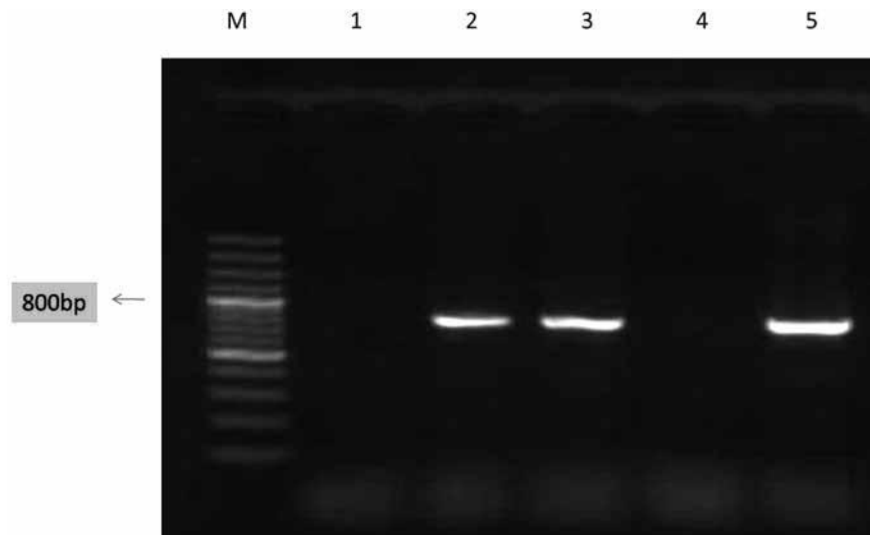

Fig. 2. PCR amplification indicating segregation of SCAR W11 with papaya sex.W11 SCAR marker giving an amplicon of 800bp size only in hermaphrodite, but not in female plants; M – 100bp ladder; 2,3,5: hermaphrodite papaya cultivar of Arka Surya Micropropagated

a cross between 'SunUp' and 'Kapoho'. Urasaki *et al.* (13) they also reported that a 450bp fragment (PSDM) present in the male and hermaphrodite plants, but absent in the female plants, with no failure after screening of 17 different individuals. Similarly, Chaturvedi *et al*. (2) reported that a 800bp amplified fragment was obtained in hermaphrodite and male papaya plants only, but absent in case of female plants. They used 84 F_1 plants of an intergeneric cross of *C. papaya* × *Visconcella cauliflora* information about the sex of which was unknown.

Observations recorded from field plants of Arka Surya and IGH proved that results from marker matched with the same. PCR screening of 50 seedlings plants (25 Arka Surya and 25 IGH) for sex expression identified 12 hermaphrodites and 13 females from Arka Surya and 10 hermaphrodites and 15 females from IGH for field planting. The results of the field validation of micro propagated Arka Surya showed that all 25 plants were hermaphrodite. This prediction corresponded with the amount of females and hermaphrodites actually observed during the blooming period in the field. So both observations recorded from field plants and results obtained from markers were same. The similar findings have also been reported previously by Deputy *et al*. (4) where they reported that a large number plants of 1,937 seedlings were screened and those plants forecasted to be hermaphrodites were transplanted in the field.

The PCR screening of SCAR T1 DNA markers showed amplification for both the female and hermaphrodite sexes of IGH 17 different cultures (Figure 3). The desired size of band (~1300bp) is present in all 17 samples. SCAR T12 DNA markers showed segregation for hermaphrodite sex of IGH 17 different cultures (Figure 4). PCR was employed on genomic DNA from IGH 17 different cultures using primers of SCART12 marker. The desired size of band (~800bp) was present in hermaphrodite plants but not in female plants among the 17 different cultures. Similar results were reported by Deputy *et al*. (4) that the molecular method, utilizing SCAR T12 and SCAR T1 as a positive control, was utilized correctly to forecast hermaphrodite papaya plants in a population of seedlings with an overall accuracy of 99.2%. Costa *et al*. (3) also established sex differentiation through ISSR marker at 500bp in *Carica* and *Vasconcellea* spp. The ISSR primer recognized only in hermaphrodite papaya samples of *SS72/12*, and absent in female seedlings.

The *in vitro* plants showed same result with regard to the expression of sex types (hermaphrodite explant yielded only hermaphrodite plants and female explant only female). Total 117 plants were planted and all bore same sex flowers as per mother culture. The observations recorded from Arka Surya and IGH micro propagated field plants concurred exactly with the results obtained from marker studies. Deputy *et al.* (4) also studied the utility of the SCARs for

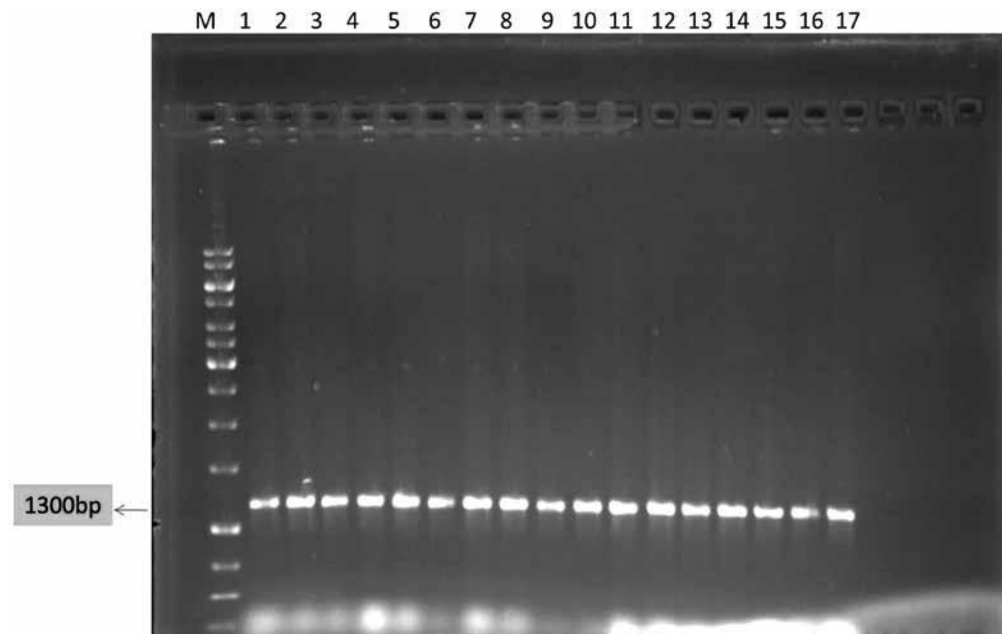

Fig. 3. PCR amplification of SCAR T1 with papaya sex. T1 SCAR marker giving an amplicon of 1300bp size in both hermaphrodite and female plants; as a positive control; M – 1 kb ladder; from 1 to 17: female and hermaphrodite papaya cultivar of IGH micropropagated.

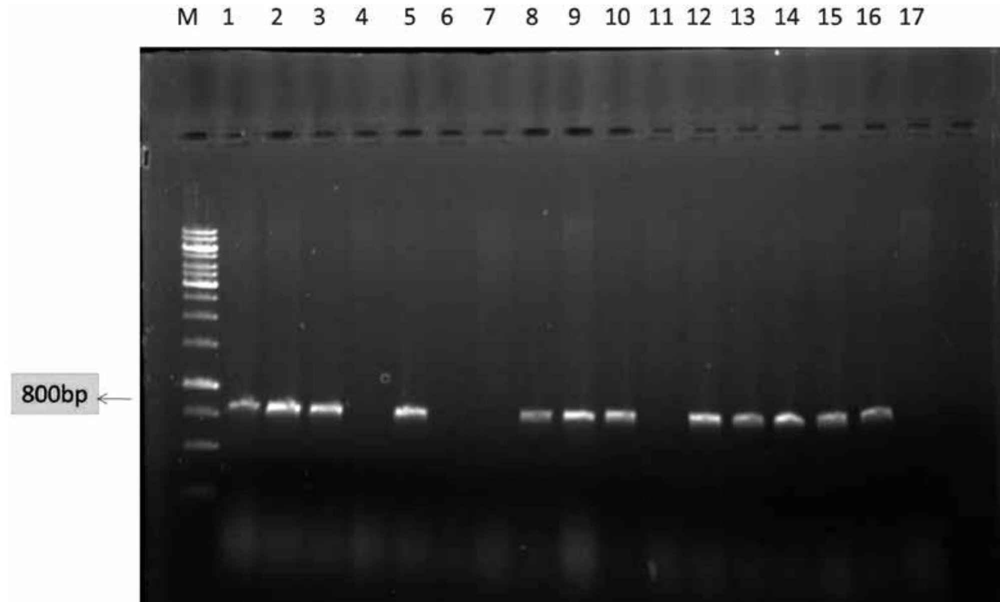

Fig. 4. PCR amplification indicating segregation of SCAR T12 with papaya sex. T12 SCAR marker giving an amplicon of 800bp size only in hermaphrodite, but not in female plants; M – 1 kb ladder; from 1 to17: female and hermaphrodite papaya cultivar of IGH micropropagated.

plant sexing prior to transplanting in the field was studied thirty five F_1 hybrids from a cross between the transgenic PRSV-resistant 'Rainbow' and either 'Laie Gold' or 'Poamoho Gold' and all 19 hermaphrodites were correctly predicted.

According to the findings derived in the present study, both markers SCAR T12 and W11 have shown same band size and same result so both markers can be used for sex expression studies in papaya. Also the results clearly indicated that plants raised *in vitro* were hermaphrodites and females without distinct variations related to sex from mother cultures maintained for 3 years. The previous study of Araya-Valverde *et al*. (1) reported same results for both primer (T12 and W11) sets permitted the accurate determination of hermaphrodite and female adult plants. Soni *et al*. (10) also reported that SCAR markers, namely, T12, CFW+CRV and W11 to be most accordant and edifying to forecast cent sex forms at seedling stage.

AUTHORS' CONTRIBUTION

Conceptualization of research (MRD); Designing of the experiments (PT, K); Contribution of experimental materials and Execution of field/ lab experiments, data collection (K, VC); Analysis of data and interpretation (K, VC); Preparation of the manuscript (K, PT, VC, NP).

DECLARATION

The authors declare no conflict of interest.

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