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Simple and robust sex determination in papaya (*Carica papaya* L.) cultivars using SCAR marker

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Abstract: For viable papaya production, more hermaphrodite plants are needed than male and female (though they produce inferior fruits). To this end, the sex of the plants must be ascertained early in their growth cycle, which is not possible with conventional methods. Molecular marker has shown its utility for this purpose, and in this study, 100 seedlings of the CO 7 gynodioecious variety and 100 seedlings of the CO 8 dioecious variety that were released from this university were analysed for their sex using the RAPD-SCAR (randomly amplified polymorphic DNA-sequence characterised amplified region) marker, T12. In those lines under investigation, the T12 marker's presence indicated male lines, while its absence indicated female or hermaphrodite lines. To confirm that there was no experimental error – that is, the lack of the SCAR marker was caused by the non-existence of the complementary target region – an oligomer primer called OPA 04 was initially employed as a control to validate the molecular marker system among the lines under investigation. Results of this study have shown that T12 has achieved 97% and 98% accuracy in correctly identifying the male and hermaphrodite flowers in the field in CO 7 and CO 8 accessions, respectively. A similarity search of the sequences of the polymerase chain reaction (PCR) product amplified by T12 from CO 7 and CO 8 has highlighted that they matched with male-specific regions of the papaya Y chromosome. Further, it was estimated that by employing molecularly certified papaya seedlings with predetermined sex, farmers could save up to 55% on labour costs when compared to approaches that use seedlings from conventional ways by implying the removal of male plants. Thus, the findings of this study help growers and breeders to identify sex early and guarantee profitable papaya production.

Keywords: male, female and hermaphrodite flowers; molecular markers; papaya; sex expression

Papaya (*Carica papaya* L.) belongs to the *Caricaceae* family, a relatively small group of herbaceous, shrubby, or tree-like plants comprising six genera (Van Droogenbroeck et al. 2002). Papaya possesses a haploid set of nine chromosomes, constituting a compact genome of 372 Mb (Liu et al. 2004). It is cultivated worldwide, especially in South Asia. In India, papaya holds a significant position as one of the most vital fruit crops, with a production of 5 227.5 million t, boasting a productivity

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of 35.6 metric t/ha from a vast area of 147 million ha (as per the Advance Estimate of 2022–2023 by the Ministry of Agriculture and Farmers Welfare, Government of India).

Papaya can be either dioecious (which means that the male and female flowers grow on different plants) or gynodioecious (with hermaphrodite flowers, that is, papaya bears both male and female flowers). In many tropical and subtropical regions, both dioecious and hermaphrodite papaya cultivars are cultivated primarily for their nutritious fruits and, to a lesser extent, for their latex (Van Droogenbroeck et al. 2002). Papaya exhibits three primary sex types: male (*Mm*), female (*mm*), and hermaphrodite (M^hm), which are determined by a single gene with three alleles (*M*, M^h, and *m*). The female is homozygous recessive (*mm*), while the male and hermaphrodite are heterozygous (*Mm*, M^hm). Combinations of two dominant alleles (*MM*, M^hM^h, M^hM) are lethal (Storey 1938).

According to Hofmeyr (1967), sex types are determined in part by the genetic balance between sex chromosomes and autosomes. Papaya sex determination has been proposed to be regulated by two primitive chromosomes, XX and XY, where XX promotes female growth, and XY promotes male development. About 10% of the Y chromosome is devoted to the male-specific genomic region, whereas in hermaphrodites, the Y^h chromosome has approximately 13% of this region (Liu et al. 2004). Hermaphrodite or female plants are generally favoured for cultivation. In contrast to hermaphrodite fruits, female papaya fruits exhibit higher crude papain yields and proteolytic activity (Madrigal et al. 1980). Hermaphrodite trees, on the other hand, yield a pyriform-shaped fruit that is more in demand.

During the cultivation process, the sex of the papaya plant can be identified 120 to 180 days after sowing by visually determining the sex of the first flower, which is labour intensive process that leads to additional cost to the papaya cultivation. Consequent to this exercise, hermaphrodite plants are preferred (and in few cases female plants are preferred even though they produce inferior fruits), and male plants are eliminated after flowering in order to maximise income.

Therefore, the practice of "roguing of male trees" is necessary to maintain the proper sex ratios for maximum productivity; but in addition to wasting 6–10% of the field space, it is inefficient in terms of time, labour, water, and nutrient use (Ming et al. 2007). Usually, 3–5 seedlings per pit are planted in order to maximise the chance of identifying the hermaphrodite plants by saving time and effort. Consequently, the cost of papaya cultivation is greatly increased owing to such additional cultivation practices.

Therefore, early and simple identification of papaya sex type would be extremely useful, and such effort will enable the planting of desirable female/hermaphrodite papaya lines and, thus, drastically increase the final yield. Recent advances in molecular marker technology have provided efficient tools for this purpose. Especially markers such as sequence characterised amplified region (SCAR) [for example, T12 and W11 (Deputy et al. 2002), NAPF-2 (Parasnis et al. 2000) and PKBT-4 (Lemos et al. 2002) that were derived from randomly amplified polymorphic DNA (RAPD)], have been successfully employed to effectively distinguish male and female/hermaphrodite plants in both dioecious and hermaphrodite varieties.

This university has crossed several parent lines, including Pusa Delicious, CO 3, CP 75, and Coorg Honey Dew, to evolve CO 7, a gynodioecious line with a great yield of 340.9 t/ha per year. Conversely, CO 8 is a dioecious variety that was developed from CO 2 and has a maximum annual yield of 230 t/ha. The main objectives of this study were designed to precisely identify hermaphrodite and female plants in both gynodioecious and dioecious varieties in the nursery itself, validate them in the field and compare the benefits of cultivating such predetermined sex lines with the regular farmer's practice. In addition to increasing papaya yield, it is anticipated that this initiative will allow a reduction in cultivation expenditures.

MATERIAL AND METHODS

Experimental material. One hundred seedlings from each of CO 7 and CO 8 varieties were used in this study. In August 2022 – September 2023, those seedlings were first raised in polybags and then moved into the main field at the College Orchard of Tamil Nadu Agricultural University, Coimbatore, India. All the investigated plants were labelled as CP-7-1 to CP-7-100 for the gynodioecious variety (CO 7) and CP-8-1 to CP-8-100 for the dioecious variety (CO 8) at the nursery. Each plant was transplanted when it was 45 days old and placed in a ~13-foot pit with

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a spacing of 2.1 × 2.1 m. Regular cultivation practices recommended for papaya by this university (https://agritech.tnau.ac.in/horticulture/horti_fruits_papaya.html) were scrupulously followed.

DNA extraction and PCR amplifications for SCAR assay. DNA extraction was carried out using a modified Dellaporta method as described by Pinar et al. (2017). One notable change involved in this method was replacing sodium acetate with a mixture of phenol : chloroform : isoamyl alcohol (25 : 24 : 1) after the initial incubation step. Additionally, RNase A was substituted with a solution comprising 50 µL of sodium acetate and 150 µL of 100% ethanol. These modifications were made to optimise the DNA extraction process, making it simpler, more effective, and consistent. Subsequently, the quantity and purity of the genomic DNA were determined using a Nanodrop Spectrophotometer (Genway Nano, Cole-Parmer, UK). The isolated genomic DNA was then loaded onto a 0.8% agarose gel, and the resulting DNA bands were visualised and documented using a gel documentation system (UVITEC gel documentation unit).

In this investigation, SCAR T12, which has successfully identified male and hermaphrodite papaya plants (Deputy et al. 2002), was used. In addition to acting as a quick validation step to verify the lack of experimental errors, RAPD marker OPA 04 was utilised to guarantee the presence of template DNA in all the investigated samples. A final volume of 10 µL was used for the polymerase chain reaction (PCR), which included 4 µL of the PCR Master mix (Smart Prime Catalogue No. 280311), 1 µL each of the T12 forward and reverse primers (100 µM), 2 µL of sterile water, and 2 µL of template DNA (50 ng/µL). The amplification process involved initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 1 min, primer annealing at 55 °C for 1 min, primer extension at 72 °C for 1.5 min, and a final extension at 72 °C for 10 min, followed by cooling to 4 °C. Following their separation on a 1.5% agarose gel, the PCR products were examined under a gel documentation system (UVITEC Essential V6 – Gel Documentation Systems). As indicated by Deputy et al. (2002), if the target band at around 800 base pairs (bp) was present, the plant type would be male; if it was absent, the plant type would be hermaphrodite and female in gynodioecious and dioecious variants, respectively. After flowering, field observations of the respective plants were compared with the marker results.

The Sanger dideoxy DNA sequencing facility (Biokart India Pvt. Ltd., Bengaluru, India) was utilised to sequence the T12 PCR products derived from gynodioecious and dioecious types. The sequencing reactions were primed using the identical primer pairs that were utilised for PCR amplification. Using nucleotide BLAST with default parameters (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome), the outcomes of paired-end sequencing were compared with the papaya-specific sequences deposited to the NCBI database. Additionally, a cost comparison between cultivation methods using molecularly certified papaya seedlings for their sex types and those using conventionally generated papaya seedlings was done.

RESULTS AND DISCUSSION

This study was designed to validate molecular marker applications in two widely cultivated papaya cultivars viz., CO 7 and CO 8 in South India.

Template DNA isolation and validation of marker system. Prior to employing molecular markers to identify the sex of papaya plants, the quality and quantity of the extracted template DNA were confirmed. The methodology employed in this investigation produced sufficient and high-quality DNA for the PCR test, according to the results of the nanodrop quantification and agarose gel electrophoresis (data not shown). Consequently, a marker assay using the RAPD marker OPA 04, was carried out, and the findings verified that the examined samples'

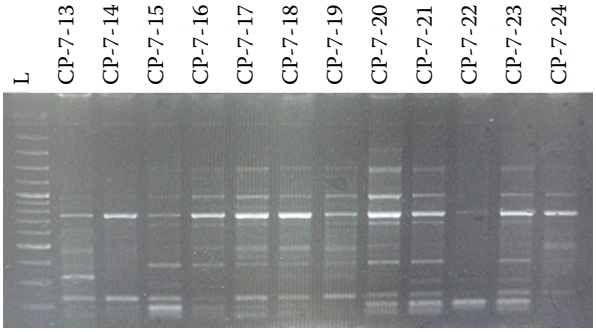

Figure 1. RAPD OPA 04 profile of the CO 7 nursery plant number 13 to 24

L – 100 bp ladder (GeneDirex Cat. No. DM003-R500); RAPD – randomly amplified polymorphic DNA; OPA 04 – oligomer primer

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DNA integrity could produce a profound RAPD profile (Figure 1).

The same template DNA employed for RAPD was used for the SCAR marker assay. This ensured that the lack of the SCAR marker, T12, was actually caused by the T12 primer(s)' complementary region not being present in the provided template DNA and not by mistakes made during experimentation.

Early sex determination in papaya cultivars CO 7 and CO 8. In this study, the sex types of juvenile papaya lines of CO 7 and CO 8 were identified using the SCAR marker, T12. Among the CO 7 plants, the male plant type was indicated by the amplification of a PCR product, which has an amplicon size of about 800 bp; the absence of this band was recorded as a hermaphrodite plant. Similarly, the presence of an ~800 bp PCR product indicated that the CO 8 plant under investigation was male. On the other hand, the plant was female because this ~800 bp band was absent (Figure 2).

Thus, the T12 marker generated an 800 bp amplification signal for male and hermaphrodite flowers, as described earlier (Deputy et al. 2002). Therefore, similar kind of sex determination were ascertained for all the 200 investigated papaya young seedlings [Tables S1 and S2 in electronic supplementary material (ESM)]. Among the investigated seedlings, it was found that 41 seedlings in CO 7 were hermaphrodite, and 38 plants in CO 8 were determined to be male (Table 1). Thus, it can be concluded that the segregation of sex-linked genes was approximately following the Mendelian segregation ratio.

Sequencing of T12 PCR product, amplified from CO 7 and CO 8. A recently evolved XY chromosome pair determines the sex of papaya, with two slightly different Y chromosomes governing the development of male (Y) and hermaphrodite (Y^h) flowers (Wang et al. 2012). A 99% identity match was found between the PCR product produced by T12 from CO 7 and CO 8 and the NCBI ID: CP010988.1, which was linked to the *Carica papaya* chromosome Y male-specific sequence (Table S1 in ESM). Furthermore, it was shown that the amplified T12 sequences from CO 7 and CO 8 had over 99% identity (Table S2 in ESM). Therefore, it is evident that the marker used in this investigation accurately captures the sequence unique to male/hermaphrodite flowers and using it in the predetermination of sex types among juvenile plants has strong validation.

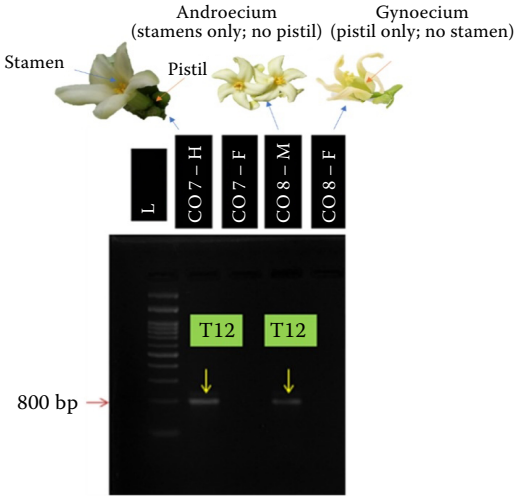

Figure 2. Agarose gel electrophoresis of PCR products amplified using the SCAR marker T12 from male (M), female (F) and hermaphrodite (H) flowers of CO 7 and CO 8

L – 100 bp ladder (GeneDirex Cat. No. DM003-R500); PCR – polymerase chain reaction; SCAR – sequence characterised amplified region

Validation of sex expression in the field. All the examined nursery papaya plants were moved into the main field in order to confirm the fact the plant sex types identified using the SCAR marker consistently displayed the same kind of sex expression while grown in the field. Of the 100 DNA samples from young CO 7 plants that were analysed, 41 samples showed PCR amplification, suggesting that hermaphrodite characteristics were present in those 41 samples. Only 38 of these plants, however, displayed hermaphrodite traits when they flowered in the field (Table S1 in ESM). Similarly, 38 DNA

Table 1. The efficiency of molecular markers in determining the sex of papaya at the nursery and its validation in the field

Serial number	Variety	Number of tested plants	Number of identified M or H plants	Number of M and H plants identified by molecular marker T12	Successful sex type identification by molecular marker T12 (%)
1.	CO 7	100	41 (H)	38 (H)	97.0
2.	CO 8	100	38 (M)	36 (M)	98.0

M – male; H – hermaphrodite

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samples from 100 young CO 8 plants exhibited expected PCR products, indicating the existence of male characteristics. However, only 36 of these plants did, in fact, exhibit male features when they flowered in the field (Table S2 in ESM). Thus, it was ascertained that the reliability of predetermination of sex in CO 7 and CO 8 cultivars using T12 marker was 97% and 98%, respectively (Table 1).

Benefits of predetermination of sex types in papaya using molecular markers. It was estimated that seedlings cost for cultivating one ha of papaya using traditional methods incurred USD 159.7 in south India (data not shown; this cost does not include other expenses like manure, pit preparation, fertilisers, irrigation, and weeding, as these were consistent for papaya cultivation by both conventional and use of predetermined sex methods). However, opting for molecularly certified seedlings with predetermined sex types cost USD 71.6 per ha (including the cost of molecular biology grade chemicals; Table 2). Therefore, by embracing molecularly certified seedlings with predetermined sex of papaya seedlings, farmers can potentially realise savings of up to 55%. The main differences between these two methods primarily revolve around the costs of seeds and the expenses associated with "roguing", or the removal of male plants by employing skilled labour. Besides, traditional methods involve additional cultivation costs (including supply of inputs such as water, fertiliser, weedicide as well as pesticide) related to raising of seedlings in the main field until they blossom. Thus, identifying and eliminating male plants early in the nursery phase optimises field space and resource utilisation. Prioritising the cultivation of female and/or hermaphrodite plants allows for customised nutrient and water management, leading to efficient resource usage, improved canopy structure, reduced competition and, finally, increased fruit production. Thus, providing specialised care and management for female and/or hermaphrodite plants streamlines maintenance and results in more effective production that leads to increased yield and profit for the farmer. Salinas et al. (2018) observed that the papaya variety BH-65 MSP with molecular sex-determining procedure initially exhibited faster growth, which translated to higher yields, although differences in height and perimeter compared to conventional sex-determining procedure gradually diminished over time.

Table 2. Cost comparison between cultivation of papaya using seedlings from conventional method and predetermined sex of papaya seedlings using molecular markers

Serial number	Components	Cost using the conventional method (USD)	Cost using molecular certified seedlings (USD)
1.	seed and seedling cost	143.2	71.6
2.	labour cost for roguing	16.5	–
Total cost		159.7	71.6

The cost involved for one ha of papaya cultivation

CONCLUSION

In summary, the early-stage sex determination in papaya using the T12 marker is a precise method that offers time and cost savings during papaya cultivation. The SCAR T12 marker effectively identified male sex expressions in both CO 7 and CO 8, with an accuracy of 97 and 98%, respectively. This advancement can significantly assist papaya growers in cultivating more female plants, ultimately leading to increased fruit production. A higher number of fruit trees per hectare will naturally result in enhanced papaya and papain production, making papaya cultivation more profitable. Thus, the findings of this study will prove beneficial for papaya breeders and growers, as it enables early-stage sex determination, contributing to a boost in papaya production.

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