Original Research Article

Evaluation of Easy and Economic Protocols for High Quality Genomic DNA Extraction and Sex Determination in Papaya using SCAR markers

ABSTRACT

Rapid and precise identification of three sex types (male, hermaphrodite and female flowers) is the key in achieving good returns in papaya (*Carica papaya L.)* cultivation. This study presents a simple, reliable, fast and cost-efficient DNA isolation protocol and a rapid and precise method of sex determination in papaya. Three DNA isolation protocols *viz.,* CTAB method, Modified Dellaporta method and BioBasic DNA isolation kit were comparatively evaluated for their simplicity, economic, DNA quantity and purity. Among them, CTAB method was regarded as relatively economic, with a cost of Rs. 11.80943 per sample with higher amount of DNA (4667.4 µg/ µl). However, it was noticed that DNA isolation with the BioBasic kit was fastest method, which took only 6 hours 7 minutes. On the other hand, relatively better DNA purity was noticed with the Modified Dellaporta method (average ratio of absorbance at 260 nm and 280 nm range between 1.8-2.0) and yielded clear, intact and distinct DNA bands. Hence, Modified Dellaporta method is suggested as an ideal DNA isolation protocol for papaya. Molecular markers (such as SCAR derived from RAPD T12, W11, NAPF-2 and PKBT-4) were employed in this study to identify the sex type and they have successfully distinguished between male and hermaphrodite plants in both dioecious and gynodioecious varieties; especially, NAPF-2 has successfully distinguished male in dioecious varieties. Evaluation of different kinds of molecular markers has shown that the SCAR marker T12 can be used as reliable marker for rapid and precise identification of papaya sex type.

Keywords: Papaya; DNA isolation method; CTAB method; Modified Dellaporta method; Sex determination; SCAR markers; Male;Hermaphrodite and Female plants

1. INTRODUCTION

Carica papayaL., native of Latin America, is a diploid (2n = 18) belonging to the small family Caricaceae with 6 genera and 35 species (Ming *et al.,* 2005). Papaya is a widely grown fruit tree in tropical and subtropical regions (Wu *et al.,* 2012). Papaya is one of the most important fruit crops of India with a production of 5227.49 million tons and productivity of 35.55 million tons per ha and grown over an area of 147.04 million ha (Advance Estimate of 2021-2023 by Ministry of Agriculture and Farmers Welfare, Government of India). It is chiefly cultivated for its edible fruits and milky latex that yields papain, a proteolytic enzyme. As the yields and proteolytic activity of the crude papain obtained from the female fruits are greater than that obtained from hermaphrodites (Madrigal *et al.,* 1980). Dioecious papaya cultivars are preferred for the extraction of papain. Moreover, it also yields higher amount of an alkaloid, carpaine, which is used as amoebacide, diuretic, thermogenic, aphrodisiac, stomachic, digestive carminative, anti - haemorrhoidal and cardiotonic (Litz, 1984). Papaya fruit is considered as a low caloric diet, rich in dietary fiber and vitamin A and C, whereas, it contains less quantity of sodium and hence regarded as perfect choice for health-conscious people (Parasnis *et al.,* 1999; Gamage *et al.,* 2003). Papaya fruits is also processed into drinks, jams, dried fruits and desserts and they become an major agricultural export commodity for developing countries. Besides its fruits, papaya leaves and flowers are also be used as vegetables (Watson 1997). Farmers generally prefers-papaya cultivation due to its fast growth, short duration and early maturity, high-yields and more importantly, its adaptability to diverse range of global environments (Evans *et al.,* 1999).

Papaya (*Carica papaya* L.) is a polygamous species with three sex types: male, hermaphrodite and female. In general, sex in papaya is controlled by a single gene having at least three alleles; a dominant allele for male plants, a different

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dominant allele for hermaphrodite plants and a recessive allele for female plants (Hofmeyr 1938; Storey 1938, 1953, 1976; Sondur *et al.,* 1996). All wild populations of papaya are dioecious (male and female plants), whereas gynodioecious (hermaphrodite and female plants) is a product of domestication (VanBuren *et al.,* 2015) and cymose inflorescences arise in axils of leaves. The type of inflorescence produced depends on the sex of the tree (Figure 1). Papaya varieties are either dioecious (with unisexual flowers with exclusive male and female plants) or gynodioecious (with bisexual and unisexual flowers i.e., hermaphrodite and female plants). There is no sexual dimorphism for vegetative traits in papaya; therefore, sex types can be identified only after the plants begin to flower.

Fig. 1. The Flowers of papaya. (A) Male flowers; (B) Female flowers; (C) Hermaphrodite flowers

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Hermaphrodite and female plants have short inflorescences with few flowers, in contrast to panicles of male plants which contain dozens of flowers and are very long and pendulous (Decraene and Smets 1999; Ming *et al.,*2007). Female plants are more valued commercially compared to male papaya plants due to their fruit production. Female plants, however, contain less flesh and produce large number of seed compared to hermaphrodite plants.

Cultivation of hermaphrodite and female plants vary depending upon the utilization of its fruits. For instance, hermaphrodite trees produce a pyriform shaped papaya fruit, which is preferred in markets, whereas, female papaya fruits are preferred for industrial purpose for extraction of papain, which is used in pharmaceutical, leather and food processing industries. Enzyme activity has been reported superior in female papaya fruits compared to hermaphrodite.

Sex determination in papaya is delayed until flowering, which takes 120-180 days. Male plants are needed for pollination as these do not bear fruit and only 5% of male plants are required for pollination in a given papaya population; consequently, the rest of the male papaya plants are discarded- to minimize the fruit production cost. Therefore, cultivation of female and hermaphrodite plants is preferred to ensure profitable papaya cultivation and male plants are chopped down after flowering stage which is less economical to the farmers. Farmers using hermaphrodites for production need to germinate a minimum of five seedlings per hill to assure there are no more than 3% of female trees. The five plants in a hill must be grown for 120-180 days until the sex is determined and undesirable sex types are rogued to ensure optimal fruit productivity. Subsequently, such process is considered to be inefficient in saving time, labor, water, nutrients and lead to competition among the plants during their early growth.

On the other hand, farmers involved in female papaya tree cultivation need to germinate four seedlings per hill to keep 5% of male trees in the field. Thus, use of female trees for fruit production lead to the loss of 6 – 10% of field space for growing male trees to pollinate the females (Ming *et al .,*2007). Further, to exercise this practice, depending on the variety, farmer has to wait 120-180 days after planting. Hence early identification of hermaphrodite and female plants at nursery level itself will save time and increase productivity in given unit area.

Recent development in molecular markers enabled such early detection and markers such as sequence characterized amplified regions (SCAR) derived from randomly amplified polymorphic DNA (RAPD) markers viz., T12, W11 (Deputy *et al.,* 2002) and PKBT-4 (Lemos *et al.,*2002) has successfully shown to identify male and hermaphrodite in dioecious and gynodioecious varieties. Similarly the marker, NAPF-2 has successfully shown to tag male papaya lines, especially in dioecious varieties (Parasnis *et al*., 2000).

Though TNAU has released several papaya dioecious and gynodioecious cultivars, there is no information on rapid and precise identification of sex in *C. papaya* variety CO-7 (which is a hermaphrodite) and CO-8 (a female plant) using molecular markers. CO-7 is a gynodioecious line developed through multiple crosses with purified parents viz., Pusa Delicious, CO-3, CP-75 and Coorg Honey Dew which yields 340.9 t/ha. On the other hand, CO-8 is a dioecious variety, derived by improving of CO.2 and can yield 200 - 230 t /ha. Hence, this study is proposed rapidly and precisely identify hermaphrodite and female in gynodioecious and dioecious plants using SCAR markers.

The fundamental requirement in use of molecular marker for this purpose is isolation of good quality DNA with relatively easy and simple procedure. Papaya, as that of other tropical plants, contain higher concentration of macromolecules such as RNA, polyphenols, polysaccharides and other secondary metabolites and thus it limits the separation and purification of DNA (Jafar Amani *et al*., 2011; Qi-Xing Huang *et al*., 2013). Isolation of plant genomic DNA can also be troublesome due to inhibitors and consequent degradation of DNA (Choudhary *et al.,* 2008). As highquality DNA is must for the molecular analysis of sex determination, a rapid, cost effective and reliable DNA extraction procedure is required. This study reports a rapid and cost-effective DNA isolation and molecular markerbased identification of dioecious and gynodioecious plants.

2. MATERIAL AND METHODS

2.1. Plant materials

Young fresh leaf samples from CO-7 and CO-8 varieties were collected and DNA extraction procedures described by Doyle and Doyle (1987), Modified Dellaporta *et al*., (1983); Pinar *et al*., (2017) introduced a Modified Dellaporta method for DNA extraction, which involved certain modifications. To enhance the efficiency and reliability of the DNA extraction process, the method incorporated a modified approach. Instead of employing sodium acetate following the incubation step, a mixture of Phenol: Chloroform: Isoamylalcohol (25:24:1) was introduced. Additionally, RNase A was replaced with a solution comprising 50 µl of sodium acetate combined with 150 µl of 100% ethanol. These adjustments aimed to optimize the DNA extraction process by improving its effectiveness and consistency and Biobasic DNA isolation kit (Molecular Biology Kit; Catalogue SK8262, USA) were compared. Disease free and healthy leaves were collected in ice box during early morning using sterile scissors or blades and brought to lab for DNA extraction.

2.2. Evaluation of Different DNA isolation protocols

The cost and execution time of the DNA isolation procedures employed in this study was assessed. The cost of each method was estimated by summing the costs of consumables (molecular grade chemicals and plasticware) used for a single extraction. The minimum time required to complete the DNA isolation was estimated for each method, including incubation, centrifugation and drying; however, the preliminary stages of weighing and maceration of plant tissue was not considered.

2.3. DNA quantification and visualization

Genomic DNA quantity and purity was measured using Nanodrop spectrophotometer. Initially 1 µl of genomic DNA was taken from the stored DNA and measured its quantity (µg/ µl), 260/280 ratio and 260/230 ratio. DNA quality was indicated by the ratio of 260/280 which was in the range of 1.8 to 2.0. The isolated genomic DNA was loaded on 0.8 % agarose gel stained with ethidium bromide to check DNA quality. The gel was run at 120 V for 30 minutes and bands were visualized and documented in the gel documentation system (UVITEC gel documentation unit).

2.4. PCR Amplifications for SCAR assay

Polymerase Chain Reaction (PCR) was carried out in a final volume of 10 µl containing PCR Master mix 4µl, Forward Primer 1 µl, Reverse Primer 1 µl, Sterile water 2 µl, Template DNA2 µl. The amplification reaction was done with initial denaturation (- 94°C for 5 mins), with 35 cycles of denaturation (-94°C for 1 min), primer annealing (- 55°C for 1 min), primer extension (- 72°C for 1.5 mins) and, final extension (- 72°C for 10 mins) then hold at 4°C. The primers used in this study listed in Table 1. Before that the annealing temperature of the above markers were optimized using gradient PCR with a temperature ranging from 50 to 65°C (Naveenaa , 2021). The PCR products were run on 1.5 % agarose gel at 120V for 2 hours to ensure the complete separation of bands. The gel was scanned through the gel documentation system (UVITEC gel documentation unit).

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Table.1. Details of markers and their respective primers used in this study

S.No	Primer	Sequence	Linked trait	T_A (°C)	Product Size	Reference
1	W11 – F	CTGATGCGTGTG TGGCTCTA (20)	Male and Heramaphroditism	55	~800 bp	Deputy *et al*., 2002
	W11 – R	CTGATGCGTGA TCATCTACT (20)	Male and Heramaphroditism			
2	T12 – F	GGGTGTGTAGGC ACTCTCCTT (21)	Male and Heramaphroditism	56	~800 bp	Deputy *et al*., 2002
	T12 – R	GGGTGTGTAGCA TGCATGATA (21)	Male and Heramaphroditism			
3	NAPF-2 – F	GAGGATCCCTATT AGTGTAAG (21)	Male	55	~831 bp	Parasnis *et al*., 2000
	NAPF-2 – R	GAGGATCCCTTTT GCACTCTG (21)	Male			
4	PKBT4 – F	GAGGGCGAGGTTT GAATTTGG (21)	Male and Heramaphroditism	55	~350 bp	Developed from RAPD sequence of Lemos *et al*., 2002
	PKBT4 – R	TTTGGTGTCCTGG TTACCCTC (21)	Male and Heramaphroditism			

3. RESULTS AND DISCUSSION

Papaya is a unique plant when it comes to flowering because it exhibits a relatively rare phenomenon. It possesses three distinct sex types, known as trioecious: female, male, and hermaphrodite. Early detection of sex in papaya cultivars at nursery stage itself greatly saves time and cost of papaya cultivation. In spite of determining the sex based on the morphological features (which may take up to 120 days in some cultivars), molecular markers have been proposed and this report used this approach.

In order to practice the marker based sex determination, it is essential to identify a rapid and simple method starting from DNA isolation to marker assay, as it is the most important limiting factor. It is obvious, that the time and cost of extraction procedure are key factors.

From this point of view, study reported here employed different DNA isolation protocol and Table 2 compares the execution time and estimated cost of each of the procedures. Among the three methods, CTAB method proposed by Doyle and Doyle (1987) can be regarded as relatively economic as it cost only Rs. 11.80per sample. The other methods are considerably more expensive, for example, Dellaporta *et al.,* (1983), Modified Dellaporta DNA extraction required Rs.

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15.68 per sample and it involved higher cost for BioBasic DNA Isolation kit Rs. 140.22 per sample. It was further noticed that when expensive reagents were used for DNA isolation protocol such as property chemicals and costly columns, the DNA isolation cost is also increased. It is essential to employ inexpensive chemicals without sacrificing the quality or quantity of DNA since it is necessary to reduce assay costs in order to sell nursery papaya seedlings at a fair price.

Table 2. Evaluation of three different DNA isolation methods with respect to execution time and cost per reaction including PCR assay

DNA Isolation method	Execution time (h:min)	Cost (Rs.) per sample
Doyle and Doyle (1987)	09:30	11.80943
Modified Dellaporta DNA isolation	10:45	15.68683
BioBasic DNA Isolation kit	06:07	140.223

Another aspect to be borne in mind is the execution time for each method, which also varied considerably: from 6 hours 7 minutes (BioBasic DNA isolation kit; considered as fastest) to 10 hours 45 minutes for Modified Dellaporta DNA isolation which is slowest; however, CTAB method took 9 hours 30 minutes to complete.

Table 3. Evaluation of efficiency of papaya genomic DNA isolation with respect to DNA purity and yield (µg/ µl) using three different protocols.

S.NO	DNA Isolation method	Avg. DNA conc. (µg/ µl)	Avg. Purity of DNA obtained (nm)	
			260/280	260/230
1	Doyle and Doyle (1987) CTAB Procedure	4667.4	2.241	1.4905
2	Dellaporta *et al.,* 1983 Modified Dellaporta DNA extraction	2388.75	1.9535	1.419
3	BioBasic DNA Isolation kit	1432.5	2.2235	1.86

It is also obvious from **Table 3** that highest amount of DNA was obtained by CTAB method (4667.4 µg/ µl). CTAB is an ionic detergent, which form an insoluble complex with nucleic acids in a low salt environment. Under these conditions, polysaccharides, phenolic compounds and other contaminants remain in supernatant and can be washed away. The DNA complex is solubilized by raising salt concentration resulting a good yield.

On the other hand, the amount of DNA obtained from Dellaporta method was 2388.75 µg/ µl whereas from DNA isolation kit was 1432.5 µg/ µl. The purity of DNA was measured by NanoDrop (Genova Nano) and it was estimated by the ratio of absorbances at A260/280. The Purity of DNA is considered to be best if it ranges between 1.8-2.0. In this study, Dellaporta method has shown to provide lower contaminated DNA as it shown the ratio between 1.8 - 2.0. Pure DNA was obtained from this method could be due to use of sodium acetate. It is highly efficient at precipitating nucleic acids and is considered as the most versatile salt because it does not inhibit many of the reactions that are often performed with purified DNAs. The salt neutralizes the charge on the nucleic acid, which causes the DNA to become less hydrophilic and precipitate it out of solution. The integrity of extracted whole genomic DNA was also analyzed by agarose gel electrophoresis and visualized in gel documentation system. Intact DNA was observed in Dellaporta DNA isolation method with clear distinct bands. In CTAB method, though higher quantity of DNA was obtained, it was found to be sheared and produced light and unclear DNA bands. On the other hand, DNA isolation kit produced no distinct bands though it has produced with A260/280 ratio higher than 2.0. (**Figure 2**)

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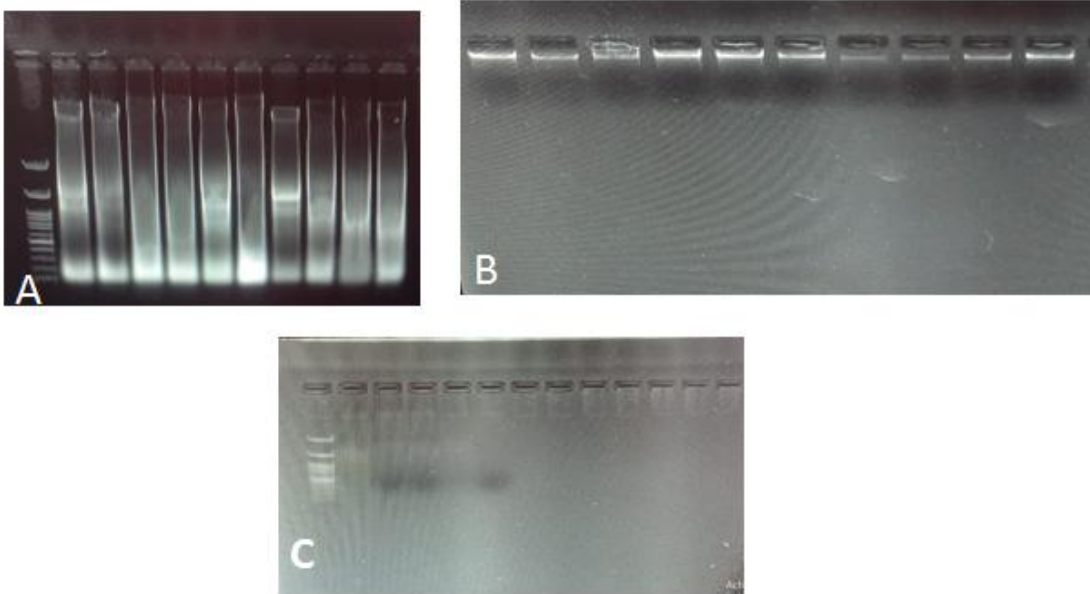
Fig. 2. Agarose gel electrophoresis of Total Genomic DNA isolation from (A) CTAB Method (B) Modified Dellaporta Method (C) BioBasic DNA Isolation kit



Though CTAB method consumed less cost and also getting higher quantity of DNA, Modified Dellaporta method resulted good quality DNA and distinct clear bands with relatively cheaper cost. Hence, Dellaporta method was used for further experiments.

Known molecular markers linked to different sex types in papaya (Table 1) were used to screen the investigated varieties (CO-7 and CO-8) to assay their expression of male, female and hermaphrodite flowers. It was found that W11 marker failed to amplify the intended PCR product from the male plants of CO-8; however, it was amplified in hermaphrodite CO-7 plants and CO-7 plants with female flowers (actually,which should not produce such band). Thus this report was not consistent with the results of Deputy *et al.,* (2002). Likewise, marker NAPF-2 did not amplify target band in male lines and thus the results of this study contradict with Parasnis *et al.*, (2000). Similarly, marker PKBT-4 did not amplify the male and hermaphrodite specific PCR product as indicated by Lemos *et al.,* (2002). Therefore, the markers W11, NAPF-2 and PKBT-4 were not used in subsequent assay to fix the sex types in papaya cultivars CO-7 and CO-8.

On the other hand, marker T12 reported by Deputy *et al.*, (2002) amplified expected PCR product in male and hermaphrodite plants and no fragment was amplified in female plants (**Figure 3**). Therefore, it was concluded that T12 primer can be utilized to identify male and hermaphrodite plants of papaya CO-7 and CO-8. As a polygamous species, papaya tends to form heterozygous population, hence DNA markers linked to sex expression in some study (for example, Parasnis *et al.*, (2000) and Lemos *et al.*, (2002)) were not successful in other lines that were evolved in different environments. However, the marker T12 reported by Deputy *et al.*, (2002) have resulted into amplification of expected PCR products with papaya cultivars CO-7 and CO-8. Thus, it is speculated that T12 marker might be located in a conserved region tightly linked to the sex expression gene at least in all these investigated papaya accessions.

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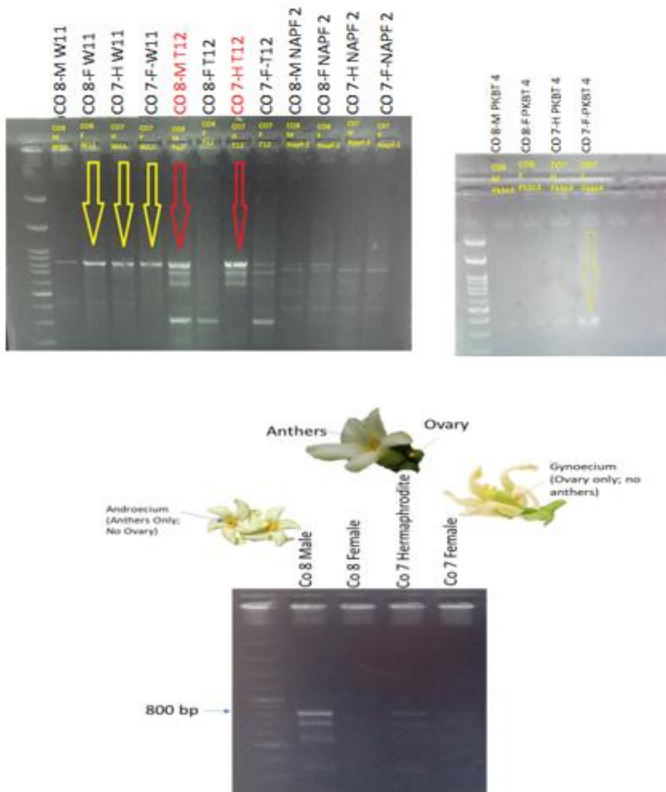
Fig. 3.Agarose gel electrophoresis of PCR products amplified from the SCAR markers- W11, T12, NAPF-2 and PKBT-4 from Male, Female, Hermaphrodite flowers of CO-7 and CO-8. M=Male: F=Female: H=Hermaphrodite



The effectiveness of molecular marker in its practical utilization largely depends on the linkage level of the marker to target character (Paterson *et al.*, 1991). Markers such as W11, NAPF-2, PKBT-4 were able to differentiate sex expression in previous reports (Deputy *et al.*, 2002 ; Lemos *et al.,* 2002 and Parasnis *et al.*, 2000). Nevertheless, in our study, these markers failed to produce the same results in male and hermaphrodite plants indicating that they are not effective in identifying sex expression of papaya, at least for the cultivar CO-7 and CO-8. However, use of the marker T12 in differentiating male and hermaphrodite plants of the investigated papaya cultivars indicated that T12 can be used as a potential marker for identification of male and hermaphrodite flower in papaya cultivars CO-7 and CO-8.

4. CONCLUSION

This study reported a rapid, cost-effective DNA isolation protocol (i.e., Modified Dellaporta method) to isolate the DNA from papaya cultivars CO-7 and CO-8 and a fast, accurate and early identification of flower sex types using a SCAR marker T12. Results of this study has great practical utility as large numbers of papaya seedlings can be screened for their sex type even at early developmental stages (i.e., before their filed planting) and hence it economically benefits the growers in planting male and hermaphrodite papaya plants in an appropriate ratio.

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