

PCR mediated detection of sex and PaLCuV infection in papaya - A review

Sangeeta Saxena*, Vijay K. Singh and Saurabh Verma

Department of Biotechnology, Babasaheb Bhimrao Ambedkar University, Vidya Vihar, Lucknow, India.

*E-mail: dr_sangeeta_saxena@yahoo.com

Abstract

The global papaya cultivation faces a major threat from various fungal and viral diseases, apart from the uncertainty in the identification of sex at juvenile stage. In case of papaya, only female and hermaphrodite plants bear fruits and the diagnostics discriminating male, female and hermaphrodite based on molecular markers used widely for sex determination, can be of great help. On the other hand, the papaya cultivation faces major challenge by leaf curl disease, which needs to be detected timely and simultaneously along with respective sex in order to achieve a higher yield. This review highlights the significance and detection of papaya sex and virus using molecular approaches; however, the authors feel that using multiplex PCR, a reliable and cost-effective technique giving results in a single attempt is by far the best approach. These molecular diagnostics may save papaya industry and give farmers a complete package of healthy (virus free) female/hermaphrodite seedling.

Key words: Geminivirus, multiplex PCR, Begomovirus, PaLCuV, coat protein.

Introduction

Papaya being polygamous in nature produces both dioecious and gynodioecious varieties. Although many methods of vegetative propagation, such as the use of cuttings, grafting and tissue-cultured materials, are available which are laborious and expensive, propagation of papaya by seed is the most practical, efficient and economical method of raising the crop till date in India. Hence, farmers or even kitchen gardeners prefer seed as the sole planting material for papaya cultivation at large scale or even for backyard gardening. Papaya flowers in three to six months after transplanting, ripe fruits are produced at the age of nine to fourteen months, so it is very difficult for the farmers to detect the sex/fruitlet crop in early stages and they have to wait for a long time from planting till harvest. Therefore, they take a long time in deciding whether to uproot the unproductive non-fruitlet bearing plant or to further maintain seedling which is a female or a productive hermaphroditic plant, for a good harvest. In this review, we want to emphasize the importance of determination of the sex type of papaya seedlings using molecular approaches prior to the flowering stage to avoid the need for removing undesired sex plant (e.g. males) from the field thus saving, labor, man power, time, fertilizer, irrigation and other resources (Magdalita *et al.*, 2003; Mishra *et al.*, 2007).

With such major economics involved in papaya production, sex determination is considered as an important subject of genetic analysis because it is directly related to commercial aspects of fruit production. A thorough literature survey revealed sex identification in papaya is governed by a single gene with three alleles *i.e.*, M for male, Mh for hermaphrodite and m for female. Females (mm) are homozygous recessive while both males (Mm) and hermaphrodites (Mhm) are heterozygous. Around 25% of seeds are nonviable in fruits because of dominant alleles found in respective combinations like MM, MMh or MhMh are embryonically lethal. Research findings suggest that M and Mh

are genetically inactive regions where vital genes are absent in case of papaya sex chromosomes (Hofmeyr, 1967; Storey, 1976). According to reports by Storey *et al.* (1976), the sex-determining region codes for a lethal factor, recombination-suppressing factor and genes regulating development of stamen and carpel. Before analyzing any approach, we need to know that in case of papaya, the sex determination is based on the XY chromosome type. Some initial studies found that although females are homogametic and males/hermaphrodites are heterogametic, heteromorphic chromosomes or unpaired chromosomes or any chromatin body has not been found in case of papaya cytological studies (Westergaard, 1958).

The traditional papaya cultivation starts with sowing of seeds in a group of three that are later (3-4 months) identified as male or female on the basis of floral buds. This whole exercise of identification of sex after flowering and further uprooting the undesired male plants, ends up in wasting a lot of time, land, manpower, irrigation and fertilization. All the wasteful practices by farmers could be avoided if sex is known prior to plantation in field so that a number of papaya plants can be strategically planted for one male seedling surrounded by nine female seedlings to maximize the production with no compromise on pollination.

Many different types of diseases infect papaya plants thus jeopardizing their production to a considerable extent resulting in gross reduction in its yield and commercial value. Many viruses like leaf curl, mosaic, ring spot *etc.* infect papaya in sub-tropical countries however, leaf curl disease in papaya is of most significance in terms of its adverse effect on plantation and financial losses. As a result, it leads to enormous loss to the growers as well as Government of India every year. Papaya Leaf Curl Virus (PaLCuV), a Geminivirus having single-stranded circular DNA is responsible for this devastating leaf curl disease (Saxena *et al.*, 1998a, 1998b and 1998c) as shown in Fig. 1. The geminiviruses belong to family Geminiviridae and is further



Fig.1. Papaya plant severely infected with Papaya Leaf Curl Virus (PaLCuV).

classified into seven genera namely Begomovirus, Mastrevirus, Topocovirus, Curtovirus, Becurtovirus, Turncurtovirus and Eragovirus. This classification is based on their genome organization and phylogenetic relationships (King *et al.*, 2012; Varsani *et al.*, 2014; Brown *et al.*, 2015). These genera share similarities in genome organization and host range including weeds and insect transmission (Hallan *et al.*, 1998). Goodman first gave the detailed description of Geminivirus in 1977 (Goodman, 1977a and 1977b). The Geminiviridae family includes virus isolates having a broad range of host plant species including both monocots and dicots. Geminiviruses are characterized by the unique geminate viral particle having fused icosahedral shaped coat protein structure. Viral genome consist of two circular single-stranded DNA (ssDNA) molecules with size ranging from 2.5–3 kb and are known as DNA-A and DNA-B respectively (Navot *et al.*, 1991; Mayo and Pringle, 1998; Saxena *et al.*, 1998c; Hanley-Bowdoin *et al.*, 1999).

Both the DNA A and DNA B components of bipartite begomovirus share an intergenic region (IR) of around 200 nucleotides (nt) in length which is highly conserved throughout the family Geminiviridae. This IR contains a nonanucleotide stem-loop structure (TAATATTAC) along with repetitive sequence-specific replicase (Rep) binding motifs called iterons and is required for replication of the viral genome (Moffat, 1997; Fauquet *et al.*, 2003).

Begomoviruses with two genomic components namely DNA-A and DNA-B are called bipartite geminiviruses as both the components are required for infection causing various diseases and are transmitted by whitefly, *Bemisia tabaci* and are reported to also have circular satellite ssDNA (Nawaz-ul-Rahman and Fauquet, 2009). There are numerous viruses that have only a single component, analogous to DNA-A, that has been isolated and shows disease symptoms alone (Navot *et al.*, 1991; Dry *et al.*, 1993 and Tan *et al.*, 1995).

Papaya cultivation in tropical and sub-tropical regions of India is severely affected by PaLCuV since past 20 years (Saxena *et al.*, 1998a, 1998b; 1998c). Therefore, we need a detection system, which can detect PaLCuV infection at juvenile stage of papaya plant so that a resistance mechanism could be developed to prevent PaLCuV from infecting papaya seedlings (Saxena *et al.*,

2011 and 2013). The strategy will ensure that the papaya field is completely free from any viral infection, in turn, minimizing the inoculum threshold. The papaya seedlings raised inside green houses or nurseries can control the vector of PaLCuV until they attain a certain height and vigor until the grown adult plants themselves become more tolerant to PaLCuV infection. Hence, a reliable, economic and rapid detection technique to detect PaLCuV is required to certify the seedlings at juvenile stage.

Sex detection in papaya: Many studies were conducted earlier using cytological, morphological, biochemical and biophysical techniques (Datta, 1971; Jindal and Singh, 1976; Sriprasertsak *et al.*, 1988) and markers but failed to accurately determine the sex in papaya plant at the seedling stage. This led to the use of DNA analysis *i.e.* DNA based molecular markers to determine sex differences as well as disease in papaya. DNA based markers have an added advantage than the others because they are consistent, unaffected by variable environment and are not stage or tissue specific. Initial studies consisted of DNA polymorphism based assay, done for amplification of random DNA fragments with single primer of arbitrary nucleotide sequence by using RAPD (Random Amplified Polymorphic DNA) because of their reliability (Williams *et al.*, 1990), developed for interspecific papaya hybrids (Magdalita *et al.*, 1997) but their use was limited because of their complexity. The highly informative microsatellite and minisatellite markers for the potential to serve as sex-specific markers in papaya were also developed (Parasnis *et al.*, 1999). However, it could only differentiate male and female and not hermaphrodite and an in depth study was needed to investigate its functional role and significance in sex determination. Next to this was the use of PCR (Polymerase Chain Reaction) based seedling sex diagnostic assay (Parasnis *et al.*, 2000) which had great commercial significance to papaya growers as well as seed companies for early identification of male/female seedlings. The RAPD was integrated with an approach known as Sequence Characterized Amplified Region (RAPD-SCAR) that amplified a 450 bp marker fragment known as PSDM (Papaya Sex Determining Marker) in order to detect the sex in papaya trees (Deputy *et al.*, 2002). The main advantage of this technique was the detection of the sex at early developmental stage thus, facilitating the cultivation and breeding of papaya by saving time, space and labor cost. The subsequent studies were carried out for the sex determination in papaya plant by using RAPD-PCR and sex diagnostic PCR assay (Deputy *et al.*, 2002; Urasaki *et al.*, 2002). Although various molecular markers have been used but the AFLP (Amplified Fragment Length Polymorphism) and SPAR (Single Primer Amplification Reaction) has proven to be a marker in producing more precise and accurate results to analyze genetic relationships among different *C. papaya* cultivars (Kim *et al.*, 2002; Saxena *et al.*, 2005).

For sex prediction in papaya, PCR-based markers have an edge over other techniques used so far that they are not affected by various external environmental conditions, have reproducibility and are cost effective. Thus, sex prediction could be done at any developmental stage of plant growth. At this point of time, PCR based technique for sex prediction in papaya is not expensive and does not need technical expertise or sophisticated equipments except for PCR machine, electrophoresis *etc.* (Magdalita and Mercado, 2003). Another approach was the use of Inter Simple

Sequence Repeat (ISSR) markers to identify genetic relationships in *Caricaceae* and sex differentiation in papaya. The objectives of this study were to analyze the genetic diversity in *Caricaceae* using ISSR markers (Costa *et al.*, 2011). To identify a specific ISSR no prior information about DNA sequences is required and being highly reproducible and co-dominant in nature many of the technical limitations imposed by other molecular markers could be successfully dealt. At the same time, one of the main disadvantages of ISSR marker was its applicability to certain limited genotypes. An experiment to differentiate papaya sex based on SAGE (Serial Analysis of Gene Expression) analysis was accomplished (Urasaki *et al.*, 2012). This technique used the idea of extracting RNA and comparing transcriptome from the flowers at two developmental stages in all the sample plants. However, this technique although very scientific in approach has its pros and cons, as it requires maturation of the flower until the attainment of developmental stages. The scientists working in the area of diagnostics have developed multiplex PCR based kits to detect the sex, diseases, transgenes *etc.* in papaya (Nageswara-Rao *et al.*, 2013). In this approach, more than one primer combinations are mixed together for PCR reaction to detect a number of amplified products thus produced, each representing the trait or character for which the PCR primers has been designed initially. This multiplex PCR based detection system seems to be the rapid and efficient technique to detect different sex in papaya in single reaction simultaneously, thus saving time and being economical as well (Hsu *et al.*, 2012).

Studies on virus detection in papaya: Plant viral disease was usually detected by various molecular techniques by the use of bioassay in which whole genome of plant pathogen can be probed compared with 2-5% of viral genome encoding antigenic determinants so it was widely used for differentiation of viral strains (Rosner and Bar-Joseph, 1984; Baulcombe *et al.*, 1986). It was sensitive, but laborious, expensive and time taking. Another method widely used for virus detection was the nucleic acid hybridization, in which the probes were designed against the target nucleic acid of pathogen. Further, combining it with PCR based detection using viral specific primers increased the sensitivity of detection as compared to the direct molecular detections. Later on, studies on mixed phase hybridization were done with target nucleic acid detection immobilized onto the solid matrix (Owens and Diener, 1981; Boulton and Markham, 1986). Another very interesting technique tissue print hybridization was used which includes printing tissue directly onto nylon and nitrocellulose membrane to localize virus. Rapidity, simplicity and sensitivity were some of the advantages of this technique (Mansky *et al.*, 1990; Chia *et al.*, 1992). Because of one or the other limitations of various above techniques, it was found that the most advanced and reliable results can be obtained by PCR. In case of PCR since the DNA sequences are amplified with high specificity using oligonucleotide primers in a simple automated reaction, it provides a good alternative to other diagnostic methods. PCR is very popular now because of its sensitivity, small sample size and reduced need of radioactive probe. Hadidi and Yang (1990) reported viroid detection by RT-PCR which was more sensitive than gel electrophoresis and ELISA (Hadidi and Yang, 1990; Hadidi *et al.*, 1993). Regardless of so many benefits, it still had some drawbacks due to the requirement of trained personnel and need of sophisticated molecular biology lab setup

(Hadidi *et al.*, 1995). Parallel to PCR, Enzyme Linked Immuno-Sorbent Assay (ELISA) was the technique frequently used; based on immunodiagnostics *i.e.* the unique recognition reaction between antigen and antibody. Using ELISA, microplate method for diagnosing antigen/plant viruses was introduced (Voller *et al.*, 1976 and 1978). This was useful in diagnosis of disease for certification and for control through eradication procedure. The popularity of ELISA is due to inherent advantages over other serological techniques. In ELISA microplate, large number of samples can be analyzed simultaneously and could be automated for high throughput applications. Further, direct tissue blot immunoassay was the single most important method for disease diagnosis as it offered great versatility (Van Regenmortel, 1982) and was very precise.

PaLCuV initially was detected by nucleic acid hybridization (Saxena *et al.*, 1998b), and later on complete sequencing and genome analysis was done (Saxena *et al.*, 1998c). This paved the way to design many more PCR primers to detect the PaLCuV using PCR. Authors are of the view that keeping in mind the isolate variability of PaLCuV, one has to select the PCR primers that are homologous to a number of PaLCuV isolates. Although multiplex PCR technique is being used to detect virus gene in transgenic papaya with reference to papaya ringspot virus, the work in case of PaLCuV is still to be carried out (Wall *et al.*, 2004; Nageswara-Rao *et al.*, 2013).

More recently, advent of isothermal nucleic acid based diagnostics like Rolling Circle Amplification (RCA) has started displacing the technically complex and resource demanding diagnostic techniques as they can be performed at low temperature ranging from 30 °C to 42 °C (Haible *et al.*, 2006). RCA, a very simple, sequence independent and very efficient technique to amplify a closed circular viral DNA from total DNA is becoming the workhorse of all virology diagnostic laboratories for the amplification of viral DNA, cloning, sequencing and identification of novel plant viruses and to study the variability (Inou-Nagata *et al.*, 2004). Thus, the viral diagnostics seem to be getting closer to a simple water bath based diagnostics, surpassing the need for expensive and sophisticated instruments and laboratory requirements. The isothermal amplification based kits are easy to handle and require less technical expertise hence amenable for on field diagnostics development in plant virology.

Currently, there are many approaches to detect papaya sex at juvenile stage and also the virus, PaLCuV that are two major bottlenecks in the growth of papaya industry. However, until now no group is working to solve the problem of sex identification and PaLCuV detection simultaneously in one single reaction. Multiplex PCR is a variant of conventional PCR, where two or more pairs of primers are used in a single reaction by simultaneously amplifying the corresponding genes. The multiplex PCR based detection method is most reliable, efficient and cost-effective and has also been successfully employed on crops. Currently many reports are pouring in where multiplex is used in quarantine to identify and detect GMOs (Genetically Modified Organisms) (Yoke-Kqueen *et al.*, 2011; Nageswara-Rao *et al.*, 2013).

A multiplex PCR based method, developed by us to identify PaLCuV and the sex of papaya plant (unpublished result) includes the isolation of plant DNA, designing of primers to detect several genes of PaLCuV along with three different papaya sexes (male, female and hermaphrodite). Finally, amplification and

careful screening of one or combination of amplified fragments may depict the presence of viral genome and a particular sex in a single tube reaction although the problem of annealing temperature being similar for all set of primers poses some problem. The diagnostics thus produced may be very useful for papaya industry for improved production. A few of multiplex PCR applications include pathogen identification, linkage analysis, gender screening, forensic studies, genetic disease diagnosis and quantitative analysis of nucleic acid templates.

Considering the significance of early identification of papaya sex and leaf curl diseases, this technique may end up in providing the farmers and papaya industry with a complete package of certified healthy (PaLCuV free) female/hermaphrodite papaya seedlings. Thus, saving the possible losses that may occur due to viral infection or uprooting of undesired male papaya plants at a later stage (by flower identification) when already time, fertilizers, irrigation and work force have been consumed. Since, multiplex PCR can be done at seeding stage with no damage to further plant growth, this technique can be advocated to nurseries, commercial plant tissue culture industries and green house facilities involved with papaya seedling propagation, multiplication and distribution to farmers.

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