ABSTRACT
The nomenclature of papaya viruses has been a source of confusion, primarily because symptomatology has been used extensively for describing papaya viruses. Although the literature is confusing and causal viruses for each of the diseases are not well identified, it appears that papaya viral diseases can generally be grouped as ring spot, mosaic and leaf curl, associated with poty, potex and geminivirus respectively.

PAPAYA LEAF CURL DISEASE
Papaya leaf curl disease was first recorded in India in 1930’s and shown to be transmitted by the whitefly *Bemisia tabaci*. The disease was characterized by typical leaf curl symptoms produced on many crop plants, by whitefly transmitted geminiviruses (WTGs). Papaya leaf curl disease is of moderate incidence and widely distributed in India. Our observation of papaya fields indicated that there has been continuous increase in the incidence of papaya leaf curl disease resulting into severe economic losses. Since whitefly is known to transmit geminiviruses and typical leaf curl symptoms on many crops are found to be associated with geminiviruses, initially on the basis of leaf curl symptoms and whitefly transmission the possible involvement of a geminivirus in papaya leaf curl disease was suspected.

Till the present work was taken up there were no reports on the detection or identification of the causal agent of papaya leaf curl disease. So, keeping in view the economic importance of the papaya leaf curl disease, an attempt was made to develop methods for the detection and identification of the causal agent.

A preferred choice for the detection of the causal agent suspected to be geminivirus was nucleic acid based diagnostics primarily on account of symptoms and transmission studies. Geminiviruses are difficult to isolate and purify due to their fragile nature. Consequently generating antibodies against these viruses for immunodiagnostic purpose is not easy. Therefore, in order to detect the casual agent of papaya leaf curl disease, which was
suspected to be a geminivirus, nucleic acid based diagnosis was considered as the method of choice.

**Nucleic Acid Hybridization Based Diagnosis**

DNA-A components of different WTGs share high nucleotide sequence identity and clones of this component have been used as general probes to detect WTGs in many crop plants and weeds. DNA-A of Indian tomato leaf curl virus (ITLCV), was, thus used as a general geminivirus probe to detect the presence of geminiviral like DNA in infected tissue. In Southern hybridization this heterologous general WTG probe gave strong signals in DNA isolated from the leaf tissue of infected as well as apparently healthy looking papaya plants collected from field, but no signals were found with DNA from the healthy plants from glass house.

Similarly, presence of geminiviral like DNA in infected plants was confirmed by using a homologous probe prepared from total DNA isolated from infected papaya. Therefore, in nucleic acid based hybridization assays geminiviral DNA probes can rapidly detect papaya leaf curl virus (PLCV) and serve as an efficient and reliable method for detecting geminiviruses.

**Polymerase Chain Reaction (PCR) Based Diagnosis**

It is reported that genome sequences that have been determined to date contain regions which are sufficiently similar for primers to be devised for use in polymerase chain reaction (PCR) test, that would detect a wide range of WTGs. Therefore, different sets of degenerate geminiviral specific as well as PLCV specific primers, were used to detect the presence of geminivirus in infected papaya. The PLCV DNA-A and B fragments of expected sizes were amplified as predicted from the annealing position of these primers with most of the WTGs genome and in case of few PLCV specific primers with PLCV genome. Further, strong positive signals of PCR amplified viral fragment by Southern blot DNA-DNA hybridization analysis with geminiviral
ITLCV DNA-A and ITLCV/TGMV DNA-B probe suggest the association of a bipartite geminivirus with leaf curl disease of papaya.

**Sequence Analysis Based Diagnosis**

DNA sequencing of PCR amplified fragments seemed to be the most precise method for identifying and characterizing geminiviruses. Therefore, PLCV DNA-A genome was cloned, sequenced and compared with genomic sequences of several WTGs from Old and New World in order to identify and also classify papaya leaf curl geminivirus. Complete nucleotide sequences of the DNA-A genome components, nucleotide sequence of the intergenic region (IR) and nucleotide and amino acid sequences of the individual ORFs were aligned to obtain all possible pairwise percent similarities and phylogenetic tree (dendrograms).

Size of the PLCV DNA-A component was found to be typical of the size of DNA-A of bipartite and single component of monopartite WTGs. Presence of conserved nonanucleotide TAATATTAC in the sequence of PLCV DNA-A served again as a diagnostic feature of geminivirus as this nonanucleotide is reported to be absolutely conserved in all the geminiviruses sequenced so far. PLCV DNA-A sequence was sufficiently homologous to sequences from other WTGs having percent similarity in the range of 66.5% to 75.8%. It has been proposed and approved by ICTV that any new isolate should be considered a strain of an already described virus if it shows more than 90% sequence identity to previously characterized virus. PLCV showing maximum homology of 75.8% (AYYV) is therefore suggested to be a distinct geminivirus.

The number, size and organization of ORFs of PLCV genome are typical of the WTGs represented by subgroup III which consist of WTGs from the Old (Asia, Africa, Australia and Europe) and the New (Americas) World with the exception of ORF AV2. This ORF of PLCV genome overlaps conserved AV1 ORF in its 3' end and is found in similar locations only in
WTGs from Old World, thus giving a preliminary evidence that PLCV is a WTG of subgroup III from Old World.

When dendrogram was constructed based on the pairwise similarity of ORF AV1 (coat protein) amino acids, two major clusters were formed, one of the New World and another of the Old World. PLCV clusters with WTGs from the Old World viruses. Maximum percent similarity of PLCV in the coat protein amino acid sequence was with ICMV (89.8%) followed by ITLCV (89%) and AYVV (80.5%) (all Old World WTGs). The amino acid sequences of the coat protein (AV1) of subgroup III viruses are more conserved than the remainder of the genome. However, a short N-terminal region (60-70 amino acids) of the coat protein is more variable than the rest of the coat protein sequence and is a close representation of the genome, and this sequence is sufficient to classify a virus isolate. The isolates that have greater than 90% identities in this region are recognized strains of the same virus species. So when N-terminal 70 amino acids of the PLCV coat protein were compared with other WTGs a percent homology range from 11.6% (MYMV) to 88.4% (ITLCV) was found, but with none of the WTG the homology reached 90% or more. These results indicate that PLCV is not a strain of already existing viruses used in the present alignment and therefore possibly a distinct geminivirus.

Intergenic region of PLCV was analyzed for the presence of repeat sequences (iterative elements) and it was found that in the region upstream to the AC1 TATA box there are three direct repeats, two of them in tandem adjacent 5’ to the TATA box. In the downstream region there is an inverted repeat. The sequence of the repeat is GGGGACNC (where N= A, T or G). This organization of iterative elements is typically similar to the viruses from the Old World. In viruses from New World no repeat is present downstream to the TATA box. A comparison and analysis of the complete nucleotide sequence and amino acid sequence of each ORF suggests that PLCV is related to the WTGs from the Old World and can be considered as a distinct geminivirus.
PAPAYA RING SPOT DISEASE
In India two aphid transmitted papaya viruses have been reported and these are referred to as papaya ring spot and papaya mosaic virus. In particular, the terms papaya mosaic and papaya ring spot have been often used to describe viruses which are transmitted non-persistently by aphids but show somewhat different symptoms. On the basis of symptoms of papaya ring spot disease, papaya ring spot virus (PRSV) a member of potyvirus group was suspected to be the causal organism.

However, the literature is quite confusing with respect to papaya mosaic disease caused by papaya mosaic virus (PMV) a potexvirus, and it is often seen that at many places papaya mosaic has been reported for papaya ring spot disease. Therefore to rule out the confusion a series of diagnostic studies were done.

Transmission and Host Range
Our transmission results indicate that the causal organism of papaya ring spot disease is both sap as well as aphid transmitted which is typical of PRSV, a potyvirus known to be transmitted by sap as well as aphids. PMV a potexvirus has been reported to be transmitted by sap and not by insect vectors, however, there are many reports that PMV can be transmitted by aphids too. PMV is one of the occasional examples in the potexvirus group which is reported to be transmitted by aphids since most potexviruses have no known arthropod vectors.

Studies on host range showed that the virus could only infect species in three dicotyledonous families (Caricaceae, Chenopodiaceae and Cucurbitaceae) which are reported for PRSV, but could not infect *Gomphrena globosa* which is a diagnostic species for PMV that produces chlorotic lesions in inoculated leaves. Our host range studies thus further indicate that we are dealing with papaya ring spot disease caused by PRSV a potyvirus and not papaya mosaic which is caused by PMV a potexvirus.
Electron Microscopy Based Diagnosis

In electron microscopy the virus particles were seen as flexuous rods about 11-12 nm wide and about 750 nm long, a characteristic of potyviruses.

Further, ultra thin section of the infected plants showed cytoplasmic inclusions pinwheels, scrolls and bundles, which is the most conspicuous and universal effect of potyvirus infection and an excellent diagnostic character of the potyvirus group. Presence of characteristic cytoplasmic inclusions confirm that the disease is caused by a potyvirus.

Immunodiagnostics

ELISA and western blots, both were used as a routine diagnostics using polyclonal PRSV antisera to detect and identify PRSV in the infected as well as apparently healthy papaya plants. Positive results in both the immunodiagnostics indicate the presence of PRSV, a potyvirus in the papaya plants showing papaya ring spot disease.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Based Diagnosis

As potyvirus sequence data are accumulating rapidly, the available sequence data made possible the development of a method for the identification of yet uncharacterized potyviruses based upon the polymerase chain reaction. Local areas of conserved amino acid sequence in the replicase and coat protein of potyviruses have been used to construct the set of degenerate oligonucleotide primers (U335 and D335) for amplification of DNA fragments on potyvirus-specific templates in a combined assay of reverse transcription and the polymerase chain reaction (RT-PCR). It is shown that the primers U335 and D335 support potyvirus specific amplification but they do not support amplification of DNA fragments on carlavirus and potexvirus tested so far. Positive amplification using such potyvirus group specific degenerate primers with infected tissue suggest that the virus causing papaya ring spot
disease is a potyvirus. RT-PCR based selective detection of potyvirus, is also a valuable extension of serological methods used in the identification of potyviruses.

In the light of the foregoing discussion following conclusions can be summarized:

**Papaya Leaf Curl Disease**

1. Based on symptoms of the infected plants, transmission by whiteflies and viral nucleic acid properties papaya leaf curl disease is suspected to be caused by a geminivirus.

2. Positive signals in nucleic acid based hybridization using both heterologous (ULCV DNA-A) and homologous geminiviral specific probe further confirm the presence of geminiviral DNA in the infected tissue.

3. PCR based amplification of the geminiviral DNA-A of expected size from infected tissue using geminivirus group specific primers confirm the role of a geminivirus tentatively called papaya leaf curl virus (PLCV). Further amplification of the complete DNA-A genome using PLCV specific primers and also a fragment of DNA-B using PLCV specific primers in combination with general geminivirus primers suggest the role of a bipartite geminivirus. Positive signals in Southern hybridization of all the PCR amplified fragments of DNA-A and DNA-B using ITLCV DNA-A as a general geminivirus probe and ITLCV/TGMV DNA-B probe respectively provide further evidence to show that papaya leaf curl disease may be caused by a bipartite geminivirus.

4. Cloning and sequencing complete DNA-A, identifying ORFs namely AV1 (coat protein), AV2 (precoat protein), AC1 (rep protein), AC2 (associated with transactivation of coat protein/movement protein genes), AC3 (replication associated protein), AC4 (determinant of symptom severity/virus movement), AC5 and comparing these ORFs
with other WTGs suggest that PLCV is a whitefly transmitted geminivirus from the Old World.

5. Complete DNA-A nucleotide sequence and coat protein N-terminal 70 amino acid sequence comparison with 17 other WTGs (both from New and Old World) suggest that PLCV is a distinct WTG from the Old World.

6. The availability of PLCV DNA-A and its use as a general geminivirus probe allows detection of uncharacterized Old World WTGs.

7. The availability of PLCV specific primers allows specific amplification and thus detection of PLCV infection in plants.

8. The availability of a well characterized coat protein gene/rep protein gene of PLCV can be used for transformation studies with a view to generate transgenic papaya plants resistant to PLCV infection.

**Papaya Ring Spot Disease**

9. Based on biological, host range and transmission studies the isolate may be a P-strain of PRSV, a potyvirus.

10. Based on molecular weight of coat protein subunits, serology and nucleic acid properties the studied isolate may be a potyvirus.

11. Electron microscopy based virus particle morphology and cytopathic studies of characteristic cytoplasmic inclusion bodies confirm the isolate under study to be a potyvirus.

12. The availability of polyclonal PRSV antibody allows detection of PRSV and other potyvirus infection in plants.

13. RT-PCR based amplification of a fragment of PRSV using degenerate potyvirus group specific primers (which do not support amplification of potexvirus) confirms the presence of PRSV a potyvirus in infected plants.

14. The availability of RT-PCR amplified fragment of 335 bp in size from the coat protein gene allows detection of PRSV infection through nucleic acid hybridization.