Detection of tomato leaf curl geminivirus in its vector *Bemisia tabaci*

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Geminiviruses are single-stranded DNA plant infecting viruses that cause major losses in important crops in tropical and subtropical countries. Tomato leaf curl virus (TLCV) belonging to the genus *Begomovirus*, is a whitefly-transmitted geminivirus that causes a severe leaf curl disease in tomato (*Lycopersicon esculentum*). The importance of this disease has prompted a great need for a rapid identification of TLCV in its hosts and vector. Polymerase chain reaction (PCR) is the most sensitive approach to detect a minute amount of viral nucleic acid. It is the most ideal method to amplify geminiviruses as they replicate via a double-stranded, circular DNA form. In this study, geminivirus specific degenerated primers were employed to detect TLCV occurring in its vector *Bemisia tabaci* by PCR based approach. One primer pair, amplified TLCV DNA fragment of about 1.1 Kb representing partly replicate gene, intergenic region and partly coat protein gene was used. When a set of primer targeted to the core region of the coat protein gene of geminivirus was used, a PCR amplified fragment of about 0.5 Kb was obtained. This approach is highly useful for an early detection of TLCV occurring in very small amount in the vector *B. tabaci*. Its implications in geminivirus management strategies and their differentiation and being discussed.

Geminiviruses belonging to family *Geminiviridae* are serious threat to vegetable and fiber crops in tropical and subtropical countries. Based on the characteristics of their genome and mode of transmission, the family *Geminiviridae* is divided into three genera, i.e. *Mastrevirus, Begomovirus* and *Curtovirus*. Begomoviruses are transmitted by the whitefly *Bemisia tabaci* and infect dicotyledonous plants. Their genome consists of two circular single-stranded (ss) DNA molecules designated as DNA-A and DNA-B. Tomato leaf curl virus (TLCV) belonging to the genera *Begomovirus*, causes a serious disease of tomato. Interestingly, it is reported to have either monocot or bipartite genome. Tomato yellow leaf curl virus (TYLCV) isolates from Israel, Italy and Spain have a single genomic molecule. However, TLCV isolates from India and TYLCV isolate from Thailand have double genomic molecule. In bipartite geminiviruses, the viral A component encodes viral functions needed for replication and encapsidation of the SS-DNA genome. It has four open reading frames (ORFs) one gene transcribed from the positive strand in leftward direction and termed as coat protein (AR1). While, four genes transcribed from the negative strand in the leftward direction and designated as AL1 (Replicase), AL2, AL3 and AL4. The coat protein (CP) gene is the most conserved gene among geminiviruses. At the amino acid level, it shows high sequence identity ranging from 93% to 95%. The CP gene product encapsidates the viral genome and has implications in the detection and identification of unknown geminivirus isolates. Sequence comparison among whitefly-transmitted geminiviruses reveals that N-terminal domain of the coat protein is variable but the core and the C-terminus domains are conserved among subgroup geminiviruses. A large intergenic region (IR) of about 300 nucleotides is located between the AR1 and AL1 genes on A component, and on B component it is present between BR1 and BL1 genes. Within the IR, there is a region named as common region (CR) which is identical in the A and B component of a geminivirus while differs to varying degrees in distinct geminiviruses.

Detection of geminivirus by conventional methods is very difficult due to low titer of virus in their vectors and hosts. Difficulty in the purification of geminivirus, non-mechanical transmission of most of them, and restricted host range make their characterization rather slow. Rapid and accurate methods are of prime importance for the detection and identification of viruses. An early detection of plant virus, however, is an important component to predict and monitor plant virus epidemics. The development of polymerase chain reaction (PCR) allows the detection of very small amounts of plant viruses in their hosts and vectors. Moreover, the PCR is the most ideal approach to detect geminivirus...
as they replicate via a circular, double-stranded, replicative form of DNA. Since PCR amplifies viral nucleic acid, this approach is extremely useful in bypassing problems associated with geminivirus serology. Here, we report the application of PCR to detect TLCV in its infected vector whitefly *Bemisia tabaci*. It is shown that the PCR approach is as sensitive as to detect TLCV in single whitefly.

**Virus culture**—Culture of tomato leaf curl virus was maintained in tomato plants *Lycopersicon esculentum* cv. Pusa Ruby under insect-proof glass house conditions. Initially, it was transmitted by a single viruliferous whitefly *Bemisia tabaci* to *L. esculentum* cv. Pusa Ruby. The symptoms consisted of curling of leaves, development of yellow mosaics and dwarving of virus infected plants with shortening of internodes, stems and petioles (Fig 1). There is reduction in fruit setting with poor size of the fruit.

**Rearing of whiteflies**—Whiteflies *B. tabaci* were reared on *Clitoria* sp. under insect proof glass house conditions.

**Inoculation of tomato plants**—Healthy whiteflies were fed on infected tomato plants (*L. esculentum* cv. Pusa Ruby) for 24 hr. Thereafter, viruliferous whiteflies were collected with the help of an aspirator and about 10 insects/plant were placed on healthy tomato plants for an additional 24 hr. The whiteflies were, then, killed by spraying the insecticides.

**Acquisition of TLCV by whiteflies**—Adult female whiteflies were given access to TLCV infected plant. After an acquisition period of 24 hr, the insects were collected by an aspirator. The insects were stored at -20°C until further processing.

**Isolation of total DNA from whiteflies**—Total DNA from viruliferous whiteflies was isolated using rapid prep genomic DNA isolation kit (Pharmacia Biotech, USA) as per the manufacturer’s instructions. Briefly, 10 viruliferous whiteflies were placed in an eppendorf tube containing 50 μl of extraction buffer (4 M Guanidium Isothiocyanate; 100 mM Tris-HCl, pH 7.5). The insects were homogenised with a plastic pestle until a uniform suspension was formed and incubated at 55°C for 30 min followed by addition of 800 μl application buffer (500 mM Tris-HCl pH 7.5). The sample was applied on a microspin column and finally the eluent containing DNA was collected. To precipitate the DNA, 80% (v/v) isopropanol was added to the eluted DNA. Finally, DNA pellets were dissolved in 50 μl TE buffer.

**Fig. 1**—Tomato leaf curl virus infected tomato plants (*Lycopersicon esculentum* cv. Pusa Ruby) showing typical symptoms.

**Polymerase chain reaction (PCR)**—Amplification of TLCV DNA fragments was done based on polymerase chain reaction using the following set of primers:

<table>
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<th>Primer</th>
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<tr>
<td>AV 494</td>
<td>GCCCTATAGTAGTATCAGAAAGCCACAG</td>
</tr>
<tr>
<td>AC 1048</td>
<td>GGGATTAGTAGAGGACATGTACGTCATG</td>
</tr>
<tr>
<td>PAL1v 1978</td>
<td>GCATCTGCAGCCCACATGTCACCTYCCNGT</td>
</tr>
<tr>
<td>PAR1c 496</td>
<td>AATACTGCAGGGCTTYCTRATACATGG</td>
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AV494/AC1048 degenerated primers corresponded to highly conserved regions of the capsid protein gene. The primers PAL1v 1978 and PAR1c 496 annealed with the AL1 ORF (codes for replication-associated protein) and the AR1 ORF (codes for the coat protein) respectively.
Fig. 2—Polymerase Chain Reaction (PCR) amplification of tomato leaf curl virus (TLCV) DNA from viruliferous whitefly vector Bemisia tabaci. A 5 μl aliquot of the 50 μl reaction mixture was analysed in 1.2% agarose gel. Lane 1= amplification of about 0.5 Kb PCR fragment with primers AV 494/AC 1048 representing TLCV coat protein; Lane 2= Lambda DNA/Eco RI and Hind III double digest marker; 3= amplification of about 1.1 Kb PCR fragment with primers PALIv 1978/PAR1c 496 representing partly replicase gene, intergenic region and partly coat protein gene of TLCV.

5 μl total nucleic acid preparation (equivalent to single viruliferous whitefly) was added to the tube containing PCR mix with a total reaction volume of 50 μl. The PCR mixture consisted of 2 μl of 2.0 mM dNTPs, 2.5 mM MgCl₂, 1 U Taq polymerase, 50 pmol of each primer (AV494/AC1048; PALIv 1978/PAR1c496). To prevent evaporation each reaction mixture was covered with 50 μl of mineral oil (Sigma, USA).

PCR was performed in Stratagene Robocycler Gradient 40 with 30 cycles. Each cycle consisted of 1 min at 94°C, 1 min 30 sec at 54°C and 1 min 30 sec at 72°C. For the last cycle, the extension time was increased to 5 min (72°C) and then temperature was decreased to 5°C until the reaction mixtures were removed.

Reaction products containing presumed PCR amplified DNA products were subjected to phenol/chloroform extraction and pelleted by adding 2.5 vol. absolute alcohol and 1/10 vol 3M sodium acetate pH 5.4. The pellets were dissolved in 50 μl sterile filtered water (Milli-Q filter). Finally, PCR amplified DNA fragments were analysed by electrophoresis on 1.2% agarose minigel (Bangalore Genci) in TAE buffer (Tris-acetate EDTA) and visualized with ultraviolet light after staining in ethidium bromide. The gel was photographed on Gel documentation System Nighthawk (pdi, USA).

All PCR reagents were procured from Bangalore Genci, India. The oligo primers AV 494/AC 1048 were synthesised by Bangalore Genci while, primers PALIv 1978/PAR1c 496 were obtained from Dr. R. L. Gilbertson (U .S. A.).

TLCV DNA from viruliferous whiteflies was amplified through PCR. As expected from the annealing position of these primers viz. PALIv 1978/PAR1c 496; and AV 494/AC 1048, PCR amplified DNA fragments of approximately 1.1 Kb and 0.5 Kb, respectively, were obtained from viruliferous whiteflies containing TLCV DNA (Fig.2). The DNA fragment of 1.1 Kb is assumed to contain part of replicase encoding gene (AL1), complete intergenic (IR) region and partly coat protein gene. While, 0.5 Kb amplified DNA fragment represents core region of the coat protein gene. No PCR amplification occurred when extracts from healthy whiteflies that had not been allowed access to infected TLCV infected leaves were used. It, thus, confirms that the PCR amplified product of 1.1 Kb and 0.5 Kb represent the TLCV genome.

Wyatt and Brown,16 and Rojas et al.17 employed the same sets of primers for the detection of geminiviruses occurring in host plants. A similar approach was used by Rybicki and Hughes18 for the detection of maize streak virus and other leaf-hopper transmitted geminiviruses. Notably, it is demonstrated here that this method is sensitive enough to detect tomato leaf curl gemivirus in single whitefly vector.

The versatility of this rapid , PCR based approach allows the detection of TLCV present in the vector whitefly. It offers several advantages and has great implications in disease forecasting programmes as well as identification and differentiation of geminivirus species. An early detection of TLCV either in the vector whitefly or in the tomato seedlings will facilitate the farmers to design strategies for preventing the disease. Before a crop starts showing geminiviral symptoms, whiteflies can be collected from a tomato growing region prone to
TLCV/geminiviral infestation and sent to the PCR equipped laboratories for proper identification of geminiviruses. DNA sequences of the PCR amplified core region of the coat protein (0.5 Kb; Fig. 2, lane 1) will facilitate TLCV detection and identification based on conserved and intervening sequences of the coat protein gene. Furthermore, restriction enzyme digestion of PCR amplified products from the coat protein gene, intergenic region and polymerase region (1.1 Kb; Fig. 2, lane 3) from individual whitefly may allow discrimination of geminivirus isolates.

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References