Duplex PCR for simultaneous detection of Begomovirus and Phytoplasma from naturally infected tomato

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ABSTRACT: Symptoms associated with the tomato leaf curl disease (ToLCD) were observed in tomato fields from Southern Karnataka, India affected with the ‘big bud’ phytoplasma. Surveys indicated incidence of tomato leaf curl disease from 27.5 to 74.3%, tomato big bud phytoplasma disease from 5.7 to 13.9% and mixed infection of leaf curl and big bud from 1.2 to 7.2% in different fields. Total DNA extracted from symptomatic and asymptomatic tomato was subjected to uniplex and duplex PCR by using Begomovirus and 16S-23SrRNA specific phytoplasma primers. Duplex PCR products of 1.2kb were obtained for 101/151 of leaf curl symptomatic tomato, 17/151 mixed infection as well as 0.5kb amplicons for 33/151 of big bud symptomatic tomato and 17/151 of mixed infection. No PCR products were observed for asymptomatic plants of 44/195. A full-length clone of Begomovirus was isolated from mixed infection and sequence analysis showed that the genome organization of this virus was found to be similar to those of other old world begomoviruses. The DNA-A molecule (2753 nt) sequences showed the highest levels of nucleotide sequence identity of 89.4 to 98.5% with the DNA-A of Tomato leaf curl New Delhi virus (ToLCNDV) isolates and less 70.2 to 86.3% identity with known tomato-infecting begomoviruses from Indian subcontinent. The 16S-23s rRNA sequences of TBB showed highest nucleotide identity of 97.6 to 99% with those of members of group 16SrII and ‘Ca. Phytoplasma australasia’, where as less than 96% with other phytoplasma groups. Duplex PCR enabled the simultaneous detection and differentiation of two different diseases. Using the duplex technique time was saved and quantity of reagents used was reduced, which translated into reduced cost of the diagnostics.

Keywords: Begomovirus, Duplex PCR, mixed infection, phytoplasma, ToLCNDV, Tomato

INTRODUCTION

Tomato (Solanum lycopersicum L.) is one of the most economically important vegetable crops and its production is constrained by tomato leaf curl disease (ToLCD), which is now widely spread in almost all tomato growing regions of the country. Begomoviruses are transmitted by whiteflies (Bemisia tabaci) and are largest and the most important groups of emerging plant viruses that cause severe diseases mainly in vegetables. Tomato leaf curl disease is caused by sixteen species of viruses belonging to the genus Begomovirus, family Geminiviridae. Based on host ranges, insect vector specificities, genome organizations and genome-wide sequence similarities, the family Geminiviridae is divided into the seven genera: begomovirus, Mastrevirus, Curtovirus, Topocuvirus, Bectovirus, Eragrovirus, Turncurtovirus, (Kings et al., 2011). In the Old World the majority of begomoviruses have monopartite genomes, although a small number have bipartite genomes consisting of DNA-A and DNA-B. The DNA-A encodes all factors required for virus replication, overcoming host defenses, insect transmission and control of gene expression, while DNA-B encodes factors required for inter- and intracellular movement in host plants (Hanley-Bowdoin et al., 2004). The majority of the monopartite begomoviruses instead associate with a newly identified class of single-stranded DNA satellites termed betasatellites (Briddon and Stanley, 2006).

Phytoplasmas are cell wall-less bacteria-like microorganisms, which are generally transmitted by leafhoppers and can inhabit and propagate in both plant and insect vectors (Li et al, 2010). In the phloem tissues of infected plants phytoplasmas are unevenly distributed and present in low titre, its detection technique should be highly sensitive. However, detection of phytoplasmas in diseased plants was difficult until the advent of molecular techniques. Observation of symptoms, insect or dodder/graft transmission to host plants, electron microscopy, commonly used diagnostic techniques etc. PCR amplification of 16S rDNA of phytoplasmas has significantly contributed to the identification and characterization of many phytoplasmas, and these molecular techniques are considered to be more sensitive than microscopic and serological methods (Bertaccini and Duduk, 2009; Rao et al., 2011). A number of phytoplasma diseases have been reported for tomato in...

Tomato in India has been affected by a complex of leaf curl and big bud, sterile big flowers, proliferation and small leaves of lateral shoots, purplish top leaves, phyllody, enlarged pistils, hypertrophic calyces and bunched type of disease. Tomato big bud was detected for the first time in tomato plants from North India (Varma, 1979). Tomato big bud phytoplasma was known to occur in different countries and was found to be caused by different phytoplasma species. Phytoplasmas, wall-less non-cultivable prokaryotes that inhabit the phloem and are naturally transmitted by leathoppers, plant hoppers or psillids (Auchenorrhyncha) have been associated with diseases from more than 700 plant species (Hogenhout et al., 2008). Virus pathogens have been commonly associated with phytoplasmas in a wide range of plant species, which suggests that phytoplasmas and viruses can naturally occur in the same plant host. Recent surveys in tomato growing areas in Southern India has indicated high incidence of Phytoplasma diseases in tomato either alone or along with leaf curl disease. The symptoms of leaf yellowing and curling, little leaf, severe stunting and phyllody were found to high incidence on tomato. Here we have developed Duplex PCR for simultaneous detection and differentiation of two different pathogens, characterized the begomovirus and phytoplasma from mixed infection symptomatic plants.

MATERIALS AND METHODS

Survey, collection of plant material and DNA extraction

Survey was conducted in 12 different tomato growing locations viz., of Hessaraghatta, Hoskote, Nelamangla, and Rajankuntae from Bangalore rural district; Chickballapur, Chinthamani, Muddnehalli, and Sidlaghatta from Chickballapur district; and Malur, Narasapura, Kolar, and Vokkaliga of Kolar district. The Symptomatic young leaves of tomato leaf curl, tomato big bud, mixed infection of leaf curl and big bud and asymptomatic samples (Fig.1) collected used for DNA extraction. Total DNA was extracted from young leaves of infected as well as healthy tomato plants; 250 mg tissue was processed for DNA extraction using modified CTAB method (Swarnalatha et al., 2013).

Uniplex PCR conditions for detection of Begomovirus and Phytoplasma

The genomic DNA isolated by the above-said method was used as template in PCR for amplification of the DNA-A genomic component of begomovirus. For PCR, one primer set, MMKBeg310F: 5’ GCGWCCRCAGATATCATATTTC’32 MMKBeg1455R: 5’GTGGATCCACATTAAAATGG 32 specific for the DNA-A component, was used for amplification. For detection of phytoplasma, phytoplasma-specific 16S rDNA universal primers P1/P7 (Deng and Hikuki, 1991) and P4/P7 (Smart et al., 1996), for the amplification of 1.7 kb and 0.5kb region consisting of the 16S rDNA, the 16S-23S spacer and the 52 end of 23S gene. All the amplifications were performed in 25µl reaction mixture containing 300ng of 1.2µl template DNA, 3 unit of Taq polymerase (sigma Aldrich USA), 25 mM MgCl2 (2 µl) 2 mM dNTPs (2 µl) 25 pmol of each of the forward and reverse primers (0.3 µl) and 10x reaction buffer (2.5 µl). The amplification was carried out using a thermal cycler (Applied Biosystems, Foster City, CA). The DNA amplification were performed with 35 cycles of denaturation for 45 sec at 94°C, primer annealing for 60 s at 55°C, and primer extension for 1 min 30 s at 72°C, with an initial denaturation at 94°C for 3 mins and a final extension for 20 min at 72°C. A final extension cycle at 72°C for 20 min was carried out to ensure the completion of amplification of all the target templates. Amplified products were visualized by agarose gel electrophoresis in the presence of ethidium bromide.

Fig 1. Tomato sample showing mixed infection of leaf curl and tomato big bud.

Duplex PCR for detection of co-infection of begomovirus and phytoplasm 

A duplex PCR assay has been developed to allow the simultaneous amplification of DNA fragments using phytoplasm- specific primers viz., P4/P7 (Smart et al., 1996) universal primer pair of 16S rDNA and begomovirus–specific (DNA-A region). PCR conditions for this duplex have been optimized ensuring the sensitive detection of the big bud phytoplasm as well as begomovirus. The total genomic DNA (300 ng) have been used to simultaneous detection of the virus and the phytoplasm. Conc. of MgCl$_2$, primer pairs and dNTPs, volume of master mix were optimized. The volume of PCR (25 $\mu$l), amount of nuclease-free water and concentration of primers (25 pmol) were standardized. Volume of 10x PCR buffer and Taq DNA polymerase was also increased. PCR master mix contained 2.5 $\mu$l of 10X PCR buffer, 2 $\mu$l of 25 mM MgCl$_2$, 2 $\mu$l of 2 mM dNTPs (Fermentas), 0.5 $\mu$l of 25 pmol of each forward and reverse primers of begomovirus and phytoplasm, 0.3 $\mu$l of Taq DNA polymerase (3 U) and 16 $\mu$l of nuclease-free water and template containing 2 $\mu$l of genomic DNA (300 ng). Nucleotide sequences of plasmid DNA from clones were determined by automated sequencing by automated DNA sequencer ABI PRISM3730 (Applied Biosystems) at Eurofins India Ltd, Bangalore, India.

Sequence analysis of viral genome DNA-A: Three clones in each case were sequenced by automated DNA sequencer ABI PRISM3730 (Applied Biosystems) at Eurofins India Ltd, Bangalore, India. Full-length sequences of begomovirus DNA A were analyzed using BioEdit version 7.0.9. Open reading frames (ORF) were predicted using GENERUNNER (Hartings Software Inc., Hastings, NY, USA; http://www.generunner.net). The sequence results of begomovirus and phytoplasm were analyzed using NCBI (www.ncbi.nlm.nih.gov) blast search, followed by multiple sequence alignments using ClustalX (Thompson et al., 1997) and Bioedit sequence alignment editor (version 5.0.9) (Hall, 1999), to determine percentage sequence similarities with other species, which showed maximum similarities in the blast search. Full-length genome of selected Begomovirus species and phytoplasm species are used for analysis. Phylogenetic trees were generated by MEGA 5.0 software (Tamura et al., 2011), using the neighbor joining method with 1000 bootstrapped replications, to estimate evolutionary distances between all pairs of sequences simultaneously.

RESULTS AND DISCUSSION
Survey and incidence of tomato leaf curl and big bud phytoplasm:

Survey conducted for tomato leaf curl disease incidence on tomato during June to September 2013, results have indicated incidence of tomato leaf curl disease was 27.5 to 74.3%, tomato big bud phytoplasm disease incidence of 5.7 to 13.9% and mixed infection of leaf curl and big bud disease 1.2 to 7.2% was noticed in different farmer’s fields in twelve locations from three districts of Karnataka based on visual symptoms. Of the 195 total leaf samples collected from 12 different locations were tested by uniplex PCR and duplex PCR. Of the 151 symptomatic samples tested by duplex PCR, 101 samples were positive to leaf curl causing begomovirus and all these samples have typical leaf curl symptoms. The 33 samples of big bud symptoms were positive to phytoplasm whereas 17 samples of mixed infection of leaf curl and big bud symptoms were positive to begomovirus and phytoplasm. The 44 asymptomatic samples tested were all negative to both begomovirus and phytoplasm (Table 1). PCR products (data not shown) of expected size 1.2 kb for begomovirus, 0.5kb and 1.2kb products were produced for phytoplasm 16S rDNA amplicons depending upon primer combination (Fig. 2) only in symptomatic samples but not in the asymptomatic plants.

PCR detection of begomovirus and phytoplasm:

Begomovirus specific primer pair designed (MMKBeg310F/MMKBeg1455R) resulted the amplification
of 1.2kb amplicon in single and mixed infections from all the symptomatic samples of leaf curl samples in uniplex PCR. The phytoplasm specific primer for amplification of intergenic region of 16S-23S rRNA, has resulted amplification of expected size PCR product of 0.5kb from individual phytoplasm affected samples as well as in mixed infection of phytoplasm and leaf curl. None of the asymptomatic samples amplify the expected PCR fragment for begomovirus or phytoplasm. The duplex PCR comprised of begomovirus specific primer pair (MKBeg310F/MKBeg1455R) and intergenic region of 16S-23S rRNA of phytoplasm when used together in the same PCR reaction tube, both pathogens begomovirus and phytoplasm were used for detection of begomovirus and phytoplasm using total DNA as a template in simplex and duplex PCR preparations. The mixed infected sample was individually amplified for begomovirus and phytoplasm in a uniplex PCR preparation to confirm the presence of both virus and phytoplasm. Further, the same sample was subjected for simultaneous detection of both in a duplex PCR preparation. The expected 1.2kb PCR amplicon specific to begomovirus in single and mixed infections, and all the symptomatic samples of big bud yielded an expected PCR amplicon of 0.5kb in single and mixed infections. As amplified in case of uniplex PCR, duplex PCR amplified begomovirus in single and mixed infection and also phytoplasm indicating duplex PCR is an efficient method for simultaneous detection of begomovirus and phytoplasm in naturally infected infections. Absence of amplification in asymptomatic samples and controls also confirms that there is no non-specific amplification. (Fig 3). The standardized duplex PCR combining primers of both begomovirus and phytoplasm were used for detection of mixed infections in all the samples collected from survey.

A partial clone of the begomovirus was obtained by polymerase chain reaction (PCR) amplification with begomovirus specific primer, cloning, sequencing and blast analysis has indicated nucleotide sequence identity of 94.5% with tomato leaf curl New Delhi virus. After confirming the begomovirus nature, complete viral genomic DNA was amplified through overlapping primers of ToLCNDV. The complete nucleotide sequence of one clone from three mixed infection samples were

Table 1. Detection of Begomovirus and Phytoplasm a infecting Tomato by duplex PCR

<table>
<thead>
<tr>
<th>District / Place</th>
<th>Total no. of Samples</th>
<th>Symptomatic samples positive to</th>
<th>Asymptomatic samples Negative to</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Begomovirus</td>
<td>Phytoplasm</td>
</tr>
<tr>
<td>Bangalore Rural Dt.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Hessaraghatta</td>
<td>18 (15)</td>
<td>10</td>
<td>3</td>
</tr>
<tr>
<td>2. Hoskote</td>
<td>20 (16)</td>
<td>11</td>
<td>3</td>
</tr>
<tr>
<td>3. Nelamangla</td>
<td>15 (12)</td>
<td>9</td>
<td>2</td>
</tr>
<tr>
<td>4. Rajankunte</td>
<td>19 (16)</td>
<td>11</td>
<td>3</td>
</tr>
<tr>
<td>Chickballapur Dt.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Chickballapur</td>
<td>13 (9)</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>2. Chinthamani</td>
<td>11 (8)</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>3. Muddnehalli</td>
<td>9 (5)</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>4. Sidlaghanna</td>
<td>21 (16)</td>
<td>11</td>
<td>3</td>
</tr>
<tr>
<td>Kolar Dt.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Murlur</td>
<td>22 (19)</td>
<td>11</td>
<td>5</td>
</tr>
<tr>
<td>2. Narasapur</td>
<td>16 (11)</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td>3. Kolar</td>
<td>19 (15)</td>
<td>9</td>
<td>4</td>
</tr>
<tr>
<td>4. Vokkaleri</td>
<td>13 (9)</td>
<td>7</td>
<td>2</td>
</tr>
<tr>
<td>Total</td>
<td>195 (151)</td>
<td>101</td>
<td>33</td>
</tr>
</tbody>
</table>

|                      | 51.79               | 16.92       | 8.72        | 22.56 | 22.56       |
Duplex PCR for virus detection

Sequence and phylogenetic analysis

The begomovirus specific amplicon of 1.2kb sequence blast analysis has indicated nucleotide sequence identity of 94.5% with tomato leaf curl New Delhi virus. The sequence obtained through amplification of genomic DNA of infected sample with overlapping primers of ToLCNDV. The complete nucleotide sequence of one clone from three mixed infection samples was determined. The complete genome sequence of DNA-A showed 91.5 to 96.5% nucleotide identity with Tomato leaf curl New Delhi virus (ToLCNDV), where as 77.1 to 86.3% homology with known tomato infecting begomoviruses from Indian subcontinent.

Sequence analysis of the begomovirus component showed the presence of a predicted hairpin structure with the sequence TAATATTAC forming part of the loop. This structure is typically part of the origin of virion-strand replication of geminiviruses. The final adenine nucleotide of the nonanucleotide sequence was by convention, used to start nucleotide numbering. Further examination of the sequences using ORF finder (http://www.ncbi.nlm.nih.gov/orffinder/orff.html) showed the presence of six predicted genes with a coding capacity greater than 12kDa, two in the virion-sense (encoding the coat protein (CP) and precoat protein AV2) and four in the complementary sense (encoding the replication associated protein (Rep), the transcriptional activator protein (TrAP), the replication enhancer protein (REN) and AC4 (of unknown function), diverging from a non-coding sequence (the intergenic region) that contains the predicted hairpin structure. This arrangement of genes is typical of the genomes (or DNA A components) of begomoviruses originating from the Old World. Phylogenetic analysis of the begomovirus sequence obtained in the study with known sequences of begomoviruses associated with ToLCNDV and selected other sequences are shown in (Fig. 4). A phylogenetic tree, based upon an alignment of the full-length sequences of DNA A component of selected begomoviruses present in Indian subcontinent, shows that the present isolate sequence grouped along with ToLCNDV. The relative positions of these isolates are well supported by bootstrapping.

BLAST search and comparisons of the 16S-23s rRNA sequences of tomato big bud showed highest nucleotide identity of 97.9 to 99% with those of members of group 16SrII and ‘Candidatus Phytoplasma australasiae’, whereas less than 96% with other phytoplasm groups. The phylogenetic analysis of different phytoplasm groups with tomato big bud phytoplasm, clustered with 16SrI and closely clustered to subgroup16SrI-D (Fig. 5). Indicating tomato big bud phytoplasm in Southern India belong to the phytoplasma group 16SrI.

In the present investigation, two different sets of primer pairs, designed to amplify begomovirus and phytoplasma were employed for successful detection of both begomovirus and phytoplasma in naturally mixed infection of tomato in duplex PCR. The modified DNA extraction protocol and various parameters, such as, primer concentration of forward and reverse primers (50-100 pmole/ìL), annealing temperature (55-60°C), and number of cycles (25-35) used, were standardized for the duplex PCR reactions for ToLCV and phytoplasma detection. Duplex PCR successfully amplified both begomovirus and phytoplasma at 55°C annealing temperature for 60 sec and 35 cycles with 100 pmole/ìL primer concentrations. This procedure can be effectively employed for validation of field samples with mixed infection.

Viruses have been commonly associated with phytoplasmas in a wide range of plant species, which suggests that phytoplasmas and viruses can naturally occur in the same plant host. These include pear and apple in New Zealand (Wood, 1997); black currant with
reversion disease in Czech Republic (Spak et al., 2004); strawberry in Czech Republic harboring Strawberry vein banding virus (SVBV), Arabis mosaic virus, strawberry latent ring spot virus, Tobacco necrosis virus, and phytoplasmas of group 16SrI, "Candidatus Phytoplasma asteris" (Fra’nova et al., 2001); malformed clovers associated with rhabdoviruses and phytoplasmas of groups 16SrI, and 16SrX, “Candidatus Phytoplasma mali” (Fra’nova et al., 2004); bleeding heart (Dicentra spectabilis), in Poland with shoot proliferation, associated with a 16SrI phytoplasma and Tobacco Rattle Virus (TRV) (Kaminska et al., 2005); the common hyacinth

**Hyacinth orientalis** L.) in Lithuania, found infected with TRV, Hyacinth mosaic potyvirus (HyMV), Arabis mosaic nepovirus (ArMV), Tobacco necrosis virus (TNV), and a 16SrI phytoplasma; and sugarcane affected by yellow leaf syndrome (YLS) in Mauritius associated with both a luteovirus (SCYLV) and a phytoplasma of group 16SrIII, X-disease (Aljanabi et al., 2001, Parmessur et al., 2002). Recently the Bunchy top symptom of Papaya was associated with phytoplasma “Candidatus Phytoplasma aurantifolia” and PRSV from Ivory Coast (Arocha et al., 2009).

A number of phytoplasma diseases has been reported for tomato in different parts of the world including Big bud, Hoja de perejil, tomato dwarf, tomato stunt, stolbur, and tomato yellows. Big bud was first described in Australia (Samuel et al., 1933) and has been reported in many tomato growing regions, including Europe (Gibb...
Broom diseases of tomato associated with phytoplasmas in Saudi Arabia belong to group II (Alhudaib, K. and Razq, A. 2011). The phytoplasma associated with tomato big bud in Brazil belongs to group 16SrIII (Amaral Mello et al., 2006). Phytoplasma diseases in tomatoes under Mauritian conditions were found to be group I and V (Gungoosingh-Bunwaree et al., 2007), whereas witches’ broom diseases of tomato associated with phytoplasmas in Saudi Arabia belong to group II (Alhudaib, K. and Razq, A., 2011). The phytoplasma associated with tomato big bud in Brazil belongs to group 16SrIII (Amaral Mello et al., 2006), and in Mexico 16SrIII and X-disease phytoplasma affecting tomato (Tapia-Tussel et al., 2010). Hoja de perejil of tomato in Bolivia, was identified as ‘Candidatus Phytoplasma lycopersici’ (Arocha et al., 2007). In China, big bud disease has been associated with phytoplasma belonging to group 16SrI and 16SrVI (Du et al., 2013, Xu et al., 2013). The Big bud and stolbur phytoplasma diseases were identified as aster yellows (16SrI) and stolbur (16SrXII-A) group in Greece (Vellos and Loliopoulou, 2007). The phytoplasma associated with tomato big bud in India belongs to group 16SrIII, based on earlier reports (Singh et al., 2012) and the present work. Although belonging to different groups, big bud phytoplasmas induce similar symptoms in tomato plants including foliar chlorosis, shoot proliferation, abnormal calyx and small leaves (Anfoka et al., 2003, Serrone et al., 2001).

Mixed infection of phytoplasmas (16SrIII, X-disease group) and two different begomoviruses (TYLCV and ToChLPV) was identified in pepper in Mexico. Phytoplasma infection was also confirmed in tomato by SEM and nested PCR assays and along with mixed infection by two begomoviruses (TYLCV and PepGMV) in Mexico (Lebsky et al., 2011). In the present study we have found association of Tomato leaf curl New Delhi Virus and Tomato big bud phytoplasma belonging to 16S group II with mixed infection leaf curl and big bud symptoms of tomato. This is the first evidence of mixed phytoplasma-begomovirus infection in tomato plants and occurrence of Tomato leaf curl New Delhi Virus on tomato in Karnataka as it is known to occur in North India (Padidam et al., 1995, Srivastava et al., 1995). The capability of coexistence of Phytoplasma with leaf curl in tomato, and the potential threat of such pathogens for the production of tomato has serious implication in the disease spread. Additional studies are needed on epidemiology, including vector migration, feeding behaviour, and survival to improve disease control.

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Duplex PCR for virus detection


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