Development of multiplex polymerase chain reaction assay for simultaneous detection of clostero-, badna- and mandari-viruses along with huanglongbing bacterium in citrus trees

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Abstract

Citrus trees harbor a large number of viral and bacterial pathogens. Citrus yellow vein clearing virus (CYCV), Indian citrus ringspot virus (ICRSV), Citrus yellow mosaic virus (CYMV), Citrus tristeza virus (CTV) and a bacterium, Candidatus Liberibacter asiaticus (CLa) associated with huanglongbing (HLB) disease, the most prevalent pathogens in citrus orchards of different regions in India and are responsible for debilitating citrusculture. For detection of these viral and bacterial pathogens a quick, sensitive and cost effective detection method is required. With this objective a multiplex polymerase chain reaction (mPCR) assay was developed for simultaneous detection of four viruses and a bacterium in citrus. Several sets of primers were designed for each virus based on the retrieved reference sequences from the GenBank. A primer pair published previously was used for greening bacterium. Each pair of primers was evaluated for their sensitivity and differentiation by simplex and mPCR. The constant amplified products were identified on the basis of molecular size in mPCR and were compared with standard PCR. The amplicons were cloned and results were confirmed with sequencing analysis. The mPCR assay was validated using naturally infected field samples for one or more citrus viruses and the huanglongbing bacterium. The mPCR assay developed here will aid in the production of virus free planting materials and rapid indexing for certification of citrus budwood programme.

1. Introduction

Citrus is one of the most important fruit crops, grown worldwide. In India, it is considered the most remunerative commercial fruit crops, grown in 10.42 million ha with a total production of 10.09 million tons (NHB, 2013). Citrus plants are hosts of many viruses and bacterial pathogens. 'Budwood' is used in propagation of commercially important citrus cultivars. Most of the viruses and HLB bacterium are transmitted by grafting or budwood (Ahlawat, 1997; Das et al., 2002; Vijayakumari et al., 2006; Prabha and Baranwal, 2011). Therefore, indexing of planting materials using a specific and sensitive detection technique is required for preventing the spread of pathogens. (Bertolini et al., 2001). Biological indexing techniques are time consuming, cumbersome and require selective healthy indicator hosts.

Enzyme-linked immunosorbent assay (ELISA) simplified virus detection and largely replaced the cumbersome graft inoculation testing for citrus viruses (EPPO, 2004). ELISA is a cost effective, robust and most commonly employed detection method for scalable to test large number of samples. However, it has many drawbacks that limit its applications for virus diagnosis for indexing (Torrans and Jones, 1981, Garney and Cambra, 1991). ELISA and its various formats require high quality antisera which are not readily available for all citrus viruses. It also lacks resolution where virus strains are closely related (Steel et al., 2010) and occurs in low concentration.

The Polymerase Chain Reaction (PCR) is a powerful technique for molecular detection of pathogens. Its high specificity and sensitivity make it the preferred tool for the detection of viral pathogens. As citrus trees are infected by several viruses and graft transmissible bacterial pathogens, simplex PCR (sPCR), which can detect only one target per reaction, will not be economical. Multiplex PCR (mPCR), where many RNA and DNA pathogens can be detected simultaneously by one reaction in a single tube, can be a more useful technique for simultaneous detection of taxonomically different pathogens infecting citrus (Wei et al., 2008).

Ito et al. (2002) developed a mPCR assay for six citrus viroids along with Apple stem grooving virus and Roy et al. (2005) also
developed a mPCR for the detection of seven citrus viruses but these did not include *Citrus yellow vein clearing virus* and HLB bacterium, which have become quite prevalent in citrus orchards of India. The primer pair used for detection of ICRSV in the mPCR was not found to be adequate, because the region selected for primer design have sequence similarity with the CYVCV sequences. The CYVCV, a recently described new mandarivirus is closely related to ICRSV in the genus *Mandarivirus* (Adams et al., 2014). These two viruses are similar in particle morphology and genome organization but share overall nucleotide sequence identity of about 74% (Rustici et al., 2002; Loconsole et al., 2012). CYMV is a *Badnavirus*, causes severe yellow mosaics disease and is widely distributed in Southern part of India, especially in sweet orange, rangpur lime and mandarins. Serological assay for CYMV are unreliable due to weak immunogenicity, therefore polymerase chain reaction (PCR) based techniques are preferable for routine detection (Ahlawat et al., 1996; Baranwal et al., 2005a,b). CTV belongs to the genus *Closterovirus* and has been responsible for one of the most destructive diseases of citrus. Infection by CTV leads to seedling yellow, quick decline and stem pitting. Serological and molecular detection methods have been used for CTV detection in India (Ghosh et al., 2008; Biswas et al., 2012). The HLB bacterium earlier known as citrus greening bacterium is an uncultured, gram-negative, walled, fastidious phloem-limited bacterium (Jagoueix et al., 1994). It caused decline or death of millions of citrus trees worldwide (Bove, 2006). HLB is present in all commercially important citrus cultivars in India (Das et al., 2002). HLB has a large geographic distribution because of vector mediated transmission by psyllid insects, *Diaphorina citri* and *Trizoa erytrea* (Capoor et al., 1967). As there is no multiplex procedure available for simultaneous detection of these viruses and the HLB bacterium we describe here the simultaneous detection of three RNA and one DNA virus along with the HLB bacterium. This mPCR assay will be highly useful in diagnosis and indexing for citrus pathogens.

2. Materials and methods

2.1. Plant materials and viruses/HLB

CYVCV infected plants of citrus cultivar, Etrog citron (*Citrus medica*), CTV infected sour lime (*C. limon*) and sweet orange plants (*C. sinensis*) and infected samples of HLB on Kinnow were collected during surveys of different citrus orchards. Bud sticks were grafted on the healthy sweet orange rootstocks and maintained in an insect free glasshouse at the Division of Plant Pathology, Indian Agricultural Research Institute New Delhi. The identity of the pathogens was confirmed through standard PCR/RT-PCR and sequencing. ICRSV infected kinnow mandarin (*Citrus reticulata*), CYMV infected sweet orange and healthy sweet orange/kinnow mandarin plants were already available in glasshouse of the IARI, New Delhi. Infected leaf samples were stored at −80 °C for further work. Mixed infections of viruses were created by bud inoculation.

2.2. Nucleic acid extraction

Total RNA was extracted from 100 mg of symptomatic as well as healthy leaf tissues of citrus plants, followed by maceration in liquid nitrogen. The commercial RNAeasy Plant Mini Kit (QIAGEN, Germany) was used for RNA isolation according to the manufacturer’s instructions with some modifications. RNA from each sample was eluted in 50 μl of RNase free water and the quality and quantity was measured with a spectrophotometer (Nanodrop, UK). The RNA from the healthy leaf tissue was used as a negative control in PCR reactions. The DNase plant mini kit (QIAGEN, Valencia, CA) was used for isolation of total DNA from leaf midribs/lamina of the HLB bacterium, CYMV infected and healthy citrus leaves tissues. The positive samples of all the four viruses and greening bacterium maintained in the glass house were used as positive control for nucleic acid extraction.

2.3. Primer design and synthesis

Several sets of primers were designed and tested during this study for detection of citrus pathogens and five sets of primers were chosen and used for standardization of simplex and multiplex PCR. The conserved gene sequences targeted for primer design were: partial coat protein (CP) gene of CYVCV and ICRSV; partial polyprotein gene of CYMV and the p18 gene of CTV using sequences retrieved from NCBI (http://www.ncbi.nlm.nih.gov/) for each virus. An earlier published primer set designed from 16S rRNA was used for detection of HLB bacterium (Harakava et al., 2000). All the nucleotide sequences were aligned using ClustalW programme as implemented in BioEdit 7.2.5.0. version. The genomic regions selected for primer design were based on the stability and compatibility. The physical properties and internal structures of primers such as hairpins, self and hetero dimers were analyzed by the OligoCalc programme (http://www.basic.northwestern.edu/biotools/oligocalc.html) (Kibbe, 2007). These primers were synthesized by Xcelris genomics, Ahmedabad, India. The primers found compatible in all combinations and highly specific and efficient for ICRSV, CYVCV, CYMV, CTV and HLB bacterium in both the formats of PCR are listed in Table 1.

2.4. Simplex PCR (sPCR)

SimplexPCR for four viruses (ICRSV, CYVCV, CTV and CYMV) and one bacterium (HLB) was performed individually using corresponding primer pairs for evaluation of primer efficiency, specificity and to determine optimal PCR conditions. For the three RNA viruses, first strand cDNA was synthesized separately using 5 μl of RNA (1 μg), 1 μl of 10 mM of antisense primer, incubated for 4 min at
70 °C, immediately chilled on ice for 5 min (as per manufacturer’s protocol with some modifications) and centrifuged briefly. Then we added 4 µl 5X First-Strand Buffer, 2 µl dNTP Mix (10 mM), 2 µl of 20 mM DTT, 2 µl (100u/µl) SMART Scribe RT (TaKaRa/Clontech, Japan) and made the volume 20 µl with RNase free water. Content of the tubes were mixed gently by pipetting and again microcentrifuged for 10s. The tubes were incubated at 42 °C for 1 h followed by 70 °C for 15 min in a thermal cycler (Model TC-E-986c, Bioer, GenePro Thermal cycler).

In sPCR, 1–1.5 µl of cDNA or DNA (200 ng) was used in a total reaction volume, consisting of 2.5 µl 10X optimized DyNazyme buffer, 0.5 µl of 10 mM dNTP, 0.25 µl (2u/ml) Taq DNA polymerase (DyNazymeml, Thermo scientific), 0.5 µl of 25 mM MgCl2, 0.5 µl of each 10 µM sense/antisense primers and sterilized distilled water to make the total reaction volume in thin-walled 200 µl tubes. The compatibility of each primer pair with other primers sets was evaluated in different combinations, using RNA or DNA extracted from plants infected with the respective pathogens. To optimize conditions, gradient PCR was performed using the following parameters; one cycle at 94 °C for 4 min, 35cycles at 94 °C for 30 s, annealing temperatures of 54/56/58/60/62°C for 35 s and 72 °C for 45 s, followed by one cycle at 72 °C for 10 min. PCR products were electrophoresed in 1.2% agarose gel and then observed under UV illumination after staining with ethidium bromide.

2.5. Multiplex PCR (mPCR)

The mPCR was standardized by synthesizing first strand cDNA for three RNA viruses using 6 µl of total RNA, mixed with 3 µl of 10 mM of the optimized reverse primers for ICRSV, CYCVV and CTV in a single tube. The mixture was incubated for 5 min at 70 °C and then the same protocol was followed as used for simplex RT-PCR using the Clontech, SMARTScribe™ reverse transcriptase kit (Clontech-639536). For optimization of mPCR various parameters were considered, including concentration of dNTPs (0.5–2 µl), MgCl2 (0–2 µl) annealing temperature (54/56/58/60/62 °C) and cDNA/DNA template volume (2–5 µl) in total volume of 25 µl reaction mixture. The mPCR assay was performed using 1-1.5 µl of cDNA of the three RNA viruses, 1.0 µl and 1.5 µl of DNA templates for CYMV and HLB bacterium respectively in 25 µl PCR reaction mixture. DyNAzYme II polymerase (Thermo scientific) and Taq polymerase (NEB – 0273S) were used. The reaction mixture contained 2.5 µl 10X optimized DyNAzYme buffer, 2.0 µl (10 µM) primer mix of each sense and antisense primers of the five pathogens in equal quantity for both forward and reverse primers separately, 1.0 µl of 10 mM dNTP, 0.5 µl (2u/µl) DyNAzYme II polymerase, 1.0 µl MgCl2 (25 mM) in a final reaction volume of 25 µl. Gradient mPCR was performed with the following parameters one cycle at 94 °C for 4 min, 35 cycles at 94 °C for 30 s, 54/56/58/60/62 °C for 35 s, and 72 °C for 45 s, followed by one cycle at 72 °C for 10 min. PCR products were analyzed by electrophoresis in a 1.5% agarose gel containing ethidium bromide and photographed under UV illumination (Biorad XR documentation system).

2.6. Sensitivity of mPCR

The total RNA and DNA isolated from different infected plants varied in concentration. To determine the sensitivity of mPCR, RNA and DNA concentrations were adjusted to 250 ng/µl and 150 ng/µl respectively (Nanodrop, UK). The first strand cDNA was synthesized with a total of 6 µl RNA mixture (2 µl RNA from each virus extract) and mixed with 3 µl of optimized reverse primers mix (10 mM) for the three viruses, and the remaining procedure was as described in the previous section. Ten fold serial dilutions (10^{-1}–10^{-10}) of DNA of three RNA viruses and DNA of CYMV and HLB were prepared using nuclease-free water and used for PCR.

2.7. Gel extraction, cloning and sequence analysis

After electrophoresis, the gel excised products were purified using Qiaquick Gel Extraction Kit (Qiagen, Germany) and ligated using the TA Cloning vector kit, (RBC USA) following the manufacturer’s instructions. The ligated plasmids were then transformed into E. coli strain DH5α (Stratagene) competent cells using standard protocol (Sambrook and Russell, 2001). Selected clones were screened and identified by colony PCR and their plasmids were isolated by GeneJET Plasmid Miniprep kit (Thermo scientific, Lithuania). The plasmids were digested with restriction enzyme Hind III (Thermo scientific, USA) for confirmation of recombinant clones. The recombinant clones were sequenced (ABI 3130 Genetic Analyzer at Xcelris Genomics Labs Ltd., Ahmedabad, India). The sequences obtained were confirmed by sequence analysis with published database available in NCBI-GenBank by the blastn programme (http://blast.ncbi.nlm.nih.gov).

2.8. Validation of multiplex RT-PCR

The optimized multiplex PCR protocol was validated by testing twenty five citrus samples exhibiting variable symptoms collected from different geographical regions of India (Table 2). The leaf samples were stored at –80 °C and subjected to mPCR to amplify the pathogen sequences. Bud sticks of symptomatic samples were grafted on healthy kinnow seedlings and maintained in the glasshouse of the Division of Plant Pathology IARI, New Delhi. Citrus plants inoculated with more than one virus or virus like disease pathogens were also tested using the developed mPCR protocol.

3. Results

3.1. Primer specificity and compatibility

Three pairs of primers for CYCVV, ICRSV and CTV and one set for CYMV were designed to amplify targeted genomic region. A single set of primers for each virus was selected based on efficient amplification and specificity of band in sPCR as well as mPCR. The best amplifications were obtained at annealing temperature ranging from 60 to 62 °C when gradient PCR was performed in both the formats. Non specific bands were amplified at suboptimal annealing temperature and density of the amplified products also varied (Fig. 2).

3.2. Optimization of simplex and mPCR

In this study initially plants were tested for single virus and bacterium infection individually by sPCR. Total RNA was isolated from symptom exhibiting plants and cDNA was synthesized using SMARTScribe™ RT (TaKaRa, Japan) and Verso Enzyme with RT enhancer (Thermo scientific). The results indicated that SMARTScribe RT was more efficient for cDNA synthesis in both sPCR as well as mPCR reactions (data not shown). The primer pairs selected for sPCR amplified DNA fragments of the expected sizes from infected plants but not from healthy plants. Amplicons of the expected sizes were obtained: 758 bp for ICRSV; 610 bp for CYMV; 451 bp for CTVV and 256 bp for CTV (Fig. 1).

Plants that were found to be positive in sPCR were used for standardizing the mPCR assay. Consistent amplification was obtained in mPCR by using 1.5–2.0 µl of cDNA of the three viruses, 1.0 µl and 1.5 µl DNA of and HLB bacterium respectively. The optimized cycling conditions was one cycle at 94 °C for 5 min followed by 35
Table 2

<table>
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<tr>
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*Symptoms observed in different field samples: YVC- Yellow vein clearing; RS- Ring spot; VF- vein flecking; M- Mosaic; Mo- Mottling; C-Chlorosis.
*mPCR reaction- Amplification (+), No amplification (−).

Fig. 1. Detection of four viruses and one HLB bacterium in citrus by simplex polymerase chain reaction using specific primer pairs. The amplified products corresponding to lanes: M: 2-log DNA ladder (0.1–10 kb, NEB); H. Healthy control; 1. 256 bp for Citrus tristeza virus (CTV); 2. 333 bp Citrus yellow vein clearing virus (CYVCV); 3. 451 bp Candidatus Liberibacter asiaticus (HLB); 4. 610 bp for Citrus yellow mosaic virus (CYMV); 5. 758 bp Indian citrus ringspot virus (ICRSV); 6. mPCR for five pathogens in a mixed sample. All the five primer pairs failed to amplify any product from healthy sweet orange used as negative control.

Fig. 2. Optimization of multiplex polymerase chain reaction for detection of four viruses and one HLB bacterium in citrus, using gradient PCR assay. Annealing temperatures were used from 54/56/58/60/62 and 64 °C (lanes 1–6, respectively) and the respective amplified products were gel electrophoresed in a 1.2% gel. Specific amplified products obtained in lanes (1–6): M: 2-log DNA ladder (0.1–10 kb, NEB); H. Healthy control; 256 bp for Citrus tristeza virus (CTV); 313 bp Citrus yellow vein clearing virus (CYVCV); 451 bp Candidatus Liberibacter asiaticus (HLB); 610 bp for Citrus yellow mosaic virus (CYMV); 758 bp Indian citrus ringspot virus (ICRSV). All of the primer pairs amplified best at 60 °C and did not amplify products in total nucleic acid from healthy sweet orange plant tissue using all five primer pairs.

3.3. Sensitivity of developed mPCR method

The sensitivity and detection limit of mPCR was determined using a ten fold serial dilution of cDNA and DNA mixtures. The highest dilution at which mPCR showed positive results was 10⁻⁶ for CYVCV, 10⁻⁸ and 10⁻⁷ for CYVCV and HLB, 10⁻⁶ and 10⁻⁵ for CTV and CYMV, 10⁻⁴ for ICRSV, respectively (Fig. 3). The results show that detection limits of mPCR gradually decrease for ICRSV, CYMV, CTV and the HLB bacterium respectively.
3.4. Detection of one or more viruses and HLB in field samples for validation of the mPCR method

In order to validate the assay twenty five samples from field and glass house were tested by the mPCR. The amplified products in different citrus samples viz., 256 bp for Citrus tristeza virus (CTV); 333 bp, Citrus yellow vein clearing virus (CYCV); 451 bp, HLB bacterium (Cla); 610 bp, Citrus yellow mosaic virus (CYMV) and 758 bp for Indian citrus ringspot virus (ICRSV) revealed the infection status of trees tested (Fig. 4). Sequencing of cloned amplicons of different viruses and HLB bacterium from different test samples showed identity with sequences of respective pathogens available in GenBank and confirmed the specific detection of these pathogens in the citrus trees. CYCV, ICRSV and HLB predominate in the northern part of India on kinnow, mandarin and sweet orange cultivars of citrus. Yellow mosaic disease caused by CYMV is common only in southern India while CTV is widely distributed throughout the country on sour lime and lemon cultivars of citrus (Table 2).

4. Discussion

Citrus yellow vein clearing is an emerging and rapidly spreading disease in many of the Asian countries wherever citrus is grown. CYCV is most closely related to Indian citrus ringspot virus. The viruses produce indistinguishable symptoms on many of the commercially cultivated citrus cultivars. Huanglongbing or citrus greening disease was first indicated from India in 1927 (Nath and Husain, 1927) and later reported from China in 1943. Now it has been reported from Asian and African countries as well as from Brazil and Florida (Bove, 2006). HLB seriously affects citrus production with the loss of millions of trees worldwide. The pathogen is transmitted by citrus psyllid, Diaphorina citri (Capoor et al., 1967). Citrus yellow mosaic virus in southern India and Citrus tristeza virus prevalent throughout the country, are other important and widely distributed diseases of citrus in India which cause significant losses (Baranwal et al., 2005a,b; Ghosh et al., 2008; Biswas et al., 2012). CTV and HLB are phloem limited pathogens and spread by insect vectors and can also be transmitted through grafting of infected budwood. Budwood used for propagation of planting material for establishing new citrus orchards, it becomes the major source of primary transmission for most of the citrus infecting viruses and greening bacterium. Therefore, quick and sensitive detection methods are essential to protect the citrus trees and prevent spreading of pathogens. ELISA is the most commonly employed method for diagnosis of plant RNA viruses while PCR based assays are employed for DNA viruses and other pathogens. PCR is the preferred and commonly employed diagnostic technique to confirm presence of plant pathogens including viruses (Boonham et al., 2014). Further, the nucleic acid based mPCR assay has the inherent capability and flexibility for simultaneous detection of viruses, bacteria and fungal pathogens (Papayannis et al., 2011; Panno et al., 2012), while ELISA lacks in such properties (Steel et al., 2010).

Many mPCR assays have been developed for field as well as horticultural crops and successfully used in detection of plant viruses (Bertolini et al., 2001; Selvarajan et al., 2011; Liu et al., 2012). For citrus also, multiplex PCR assay was developed by Ito et al., 2002 for six viroids and one Ampelovirus and Roy et al., 2005 for simultaneous detection of seven viruses. In their study they did not include huanglongbing bacterium and recently characterized, rapidly spreading Citrus yellow vein clearing virus. The multiplex PCR developed in the present study was standardized for simultaneous detection of two Mandarivirus (CYCV and ICRSV), one Closterovirus (CTV), one Badnavirus (CYMV) and huanglongbing bacterium infecting citrus in India. The primer set used for detection of ICRSV in multiplex reaction developed by Roy et al., 2005 failed to distinguish this virus from the closely associated member CYCV of the genus Mandarivirus, characterized recently from India, Turkey and China (Ahlawat and Pant, 2003; Loconsole et al., 2012). Both of the mandariviruses produce ringspot symptoms and are also similar in genome organization and virus particle structure. Therefore virus specific primers were designed and used to distinguish ICRSV and CYCV based on the expected amplicon size. The use of more than one primer pair in mPCR increases the chance of self annealing, primer dimer formation and of non-specific amplification (Brownie et al., 1997). However, we did not observe self annealing and dimerization in our protocol for mPCR. The primers for ICRSV, CYCV, CYMV, CTV and citrus greening bacteria were designed with a nearly identical annealing temperature and easily distinguishable product size in mPCR. The efficiency of multiplex PCR is determined by specific primers, suboptimal buffer constituents and annealing temperature (Elnifro et al., 2000; Ge et al., 2013; Xie et al., 2009). The primer pairs selected for each virus and bacterium consistently amplified the expected size target genomic regions. The published sets of primers, except for the HLB bacterium, were inadequate for use in mPCR protocol development because of the product size, primer interference and non specific amplification. The combinations of primers chosen for mPCR based on amplification efficiency were able to amplify the targeted genomic regions of different pathogens in the mixture. Many viruses and bacterium are difficult to detect because of low titer, uneven distribution and tissue localization in citrus plant. For the HLB bacterium and CYMV, DNA was isolated from the midrib including some portion of petiole of citrus leaves. RNA was extracted for RNA viruses from leaf tissues, using commercially available RNasey plant mini kit (QiAGEN) to reduce the unwanted impurities for better results. Our PCR standardization results revealed that Clontech, SMARTScripte RT enzyme was more efficient than Verso Enzyme with RT enhancer (Thermo scientific) for cDNA synthesis. In mPCR amplification two conventional PCR kits were used and the Thermo scientific, DynAzyme II polymerase kit produced expected amplicon size and better results in mPCR. The additional and increased concentration of MgCl2 improved amplification and efficiency of mPCR as was shown previously for the amplification of HLB and CYMV (Baranwal et al., 2005a,b). The naturally infected samples collected from different parts of the country including Kinnow, sweet orange (Malta, blood red, Pineapple etc.), lemon, grapefruit etc., were subjected to mPCR for validation of the developed method. Most of the samples were infected by more than one pathogen in mixed infections and CYCV was found to be most prevalent pathogen in the tested samples.
The mPCR assay we developed will help in sensitive and robust detection of prevalent viral and bacterial pathogens of citrus occurring in India. Multiplex PCR methods will also help in production of disease-free planting materials, indexing of mother stock and large scale indexing of field samples in test laboratories as well as in quarantine applications.

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