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Original Research Article

Molecular Techniques for Detection of *Citrus tristeza* Virus Affecting *Citrus* species of Vidarbha Region in Maharashtra, India

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A B S T R A C T

Citrus tristeza virus is one of the most important and devasting pathogen affecting citrus species worldwide, which has destroyed more than one million citrus trees in India till date. Under field conditions, diagnosis of CTV is very difficult as no visible symptoms developed. Field surveys were carried out in 2 different citrus cultivation regions of Vidarbha at two different stages in 2016-17 and CTV symptomatic leaf samples were collected from three different citrus species. Samples were tested for the presence of Citrus tristeza virus (CTV) by polymerase chain reaction with ease of universal primer T-36, showed 672 bp amplicon size and also with 10 different designed, part of coat protein gene specific primers, showed different amplicon size. These designed primers were then validated by using the other citrus spp. virus, no amplification was observed in the other isolates of virus except CTV, confirmed that designed primers are the gene specific and can be used for the screening of CTV infected plant for the production of CTV free planting material for citrus industry. The results revealed that potentially severe isolates of CTV are present in both selected major citrus cultivation regions of Vidarbha in Maharashtra.

Keywords

Cultivation, Symptomatic, Polymerase, Universal, Amplicon

Introduction

Citrus is considered to be one of the most remunerative fruit crops of India, having a lasting niche in the international trade and world finance so the global problem of citrus decline warranted special attention of agricultural scientists during the past few decades.

Tristeza disease caused by Citrus tristeza virus (CTV) is one of the most destructive and economically important viral diseases of commercial citrus worldwide (Bar-Joseph *et al.*, 1989; Lee and Rocha-Pena, 1992; Rocha-Pena etal., 1995). The disease is distributed in most citrus growing regions of the world, including Turkey.

CTV belongs to the genus Closterovirus in the family Closteroviridae (Bar-Joseph *et al.*, 1979; Bar-Joseph and Lee, 1990). It has long thread-like, flexuous, filamentous particles that are about 2000 nm by 11 nm (Bar-Joseph *et al.*, 1979; Bar-Joseph and Lee, 1990). Virions consist of 1 single-stranded, positive-sense RNA molecule encapsidated with 2 capsid proteins (CP).

The 25 kDa major CP encapsidates about 95% of the genome, and the remaining portion of the genome is encapsidated by the 27 kDa minor CP on one end of the virion (Febres *et al.*, 1996).

CTV isolates cause a variety of symptoms in citrus, depending on the scion and rootstock combination.

Mild vein clearing (M), seedling yellows (SY), quick decline (QD), and stem pitting in grapefruit (SP-G) and in sweet orange (SP-O) are several symptoms caused by different isolates of CTV. A standardized set of citrus indicator plants has been established to determine the biological characteristics of CTV isolates (Garnsey *et al.*, 1987b).

CTV strains are traditionally characterized based on symptoms induced in differential indicator hosts (McClean, 1977; Garnsey *et al.*, 1987a).

The common indicator is Mexican lime (C. aurantifolia {Christm.} Swingle) in which vein clearing, leaf cupping, and stem pitting symptoms were induced in accordance with the severity of CTV isolates (Garnsey *et al.*, 1987b).

Although biological indexing is a reliable method and provides important information, it is costly and time consuming, requiring 12-15 months under ideal conditions; therefore, a molecular assays, including and reverse transcription-polymerase chain reaction (RT-PCR) (Nolasco *et al.*, 1993) have been used to rapidly detect CTV.

The present study surveyed two different citrus growing regions of Vidarbha, and the presence of CTV was detected in three different commercial citrus species by Polymerase chain reaction and validated by using other citrus spp. virus isolates.

Materials and Methods

Field survey and sample collection

Citrus Tristeza virus symptomatic leaf samples were collected from two citrus producing areas of Vidarbha, including the Akola (AICRP, Citrus Dr.PDKV, Akola), and Warud region (Tembhurkheda) at two different stages adult and nursery stage respectively, were surveyed during the cool seasons (November-April) in 20016-17, from Orange, sweet orange and lemon. Citrus plants were visually examined and leaf samples were collected from symptomatic trees, which showed tristeza-like symptoms, such as stunting, leaf cupping, yellowing and vein clearing.

Reverse Transcriptase polymerase chain Reaction (RT-PCR)

Total RNA was isolated from 100 mg of symptomatic leaf tissue using TRI Reagent method (Sambrook *et al.*, 1989) from all three samples collected from field at two different stages. For negative control the total RNA was isolated from a healthy plant.

The effendorf thermal cycler was programmed for cDNA synthesis at 42 °C for 60 min, followed by primer extension at 72 °C for 1 min, and final extension at 72 °C for 5 min. PCR products were separated by electrophoresis in 1% agarose gel, stained with ethidium bromide, and analyzed with a Doc- It gel imaging system (GENE, Bangalore).

Confirmation of presence of CTV

The incidence of CTV was verified by PCR using PCR T-36 CP specific forward (5'ATGGACGACGAAACAAAG AAATTG3') and reverse (5'TCAACGTGT GTTGAATTTCCCA3') primers. Along

with three different field samples, a healthy sweet orange sample were used as a healthy control (HTC) and one non treated control (NTC) without any DNA sample used, to avoid the false positive result.

Detection of CTV by using new designed coat protein gene specific primers

There are 12 new different primers are designed specific to coat protein gene of CTV and used for the detection of CTV in the collected field sample of Citrus Spp. in present study. All primers given nomenclature as BTC-CTV-1, BTC-CTV-2, BTC-CTV-4, BTC-CTV-5, BTC-CTV-7, BTC-CTV-8, BTC-CTV-9, BTC-CTV-10, BTC-CTV-11 and BTC-CTV-12.

Validation of CTV CP gene specific primers with other major viruses of Citrus spp.

To avoid the unspecific amplification from new designed CTV CP gene specific primers, are validated using the other major viruses of Citrus Spp. *viz*..Citrus ring spot virus, Citrus mosaic virus, Exocortis Virus and Citrus crinkly leaf virus.RNA was isolated from symptomatic leaf tissue. The c-DNA was synthesized using random hexamer primer and PCR was carried out using all designed primers by using c-DNA from these four viruses as a template in PCR reaction.

Results and Discussion

Field survey and sample collection

In field condition, samples are collected from Citrus plant spp. which showed severe infection by, detecting with visual observation of major symptoms of CTV in Citrus Spp. *viz*.., leaf cupping, yellowing, stems pitting and vein clearing (Plate 1).

RNA isolation and C-DNA synthesis

Total RNA extracted from the CTV infected Citrus Spp. leaves was used as template for RT-PCR analysis. First strand c-DNA was synthesized from total RNA in 20ul reaction using random hexamer primer and Moloney Murine Leukemia Virus Reverse Transcriptase (MMuLV-RT).

Confirmation of presence of CTV

A 672-bp DNA fragment corresponding to the CP of CTV was amplified from the all three different samples by using T-36 gene specific forward and reverse primers, but not from the healthy control and non treated control (Plate 2) confirmed that virus are present in all three collected leaf samples of Citrus Spp.

Detection of CTV by using new designed coat protein gene specific primers

For detection of CTV, Polymerase chain reaction was carried out using above 12 mentioned primers. Out of 12 only 10 primers are showed amplification and the amplicon size (Table 2) of these primers were different (Plate 3) because primers are designed specific to part of coat protein gene. No amplification was observed in healthy and non treated control, confirmed that

Validation of CTV CP gene specific primers with other major viruses of Citrus spp.

To validate the primers, PCR was carried out by using primers BTC-CTV-1, BTC-CTV-2, BTC-CTV-4, BTC-CTV-5, BTC-CTV- 6, BTC-CTV-7, BTC-CTV-8, BTC-CTV-10, BTC-CTV-11 and BTC-CTV-12. All these primers showed amplification in CTV isolates only but no amplification was

observed in other viruses with healthy control and non-treated control (Plate 4).It conclude that, all designed primers amplifies only CTV isolates from citrus field so they are gene specific.

Table.1 Amplicon size showed by 10 new designed CTV CP gene specific primers

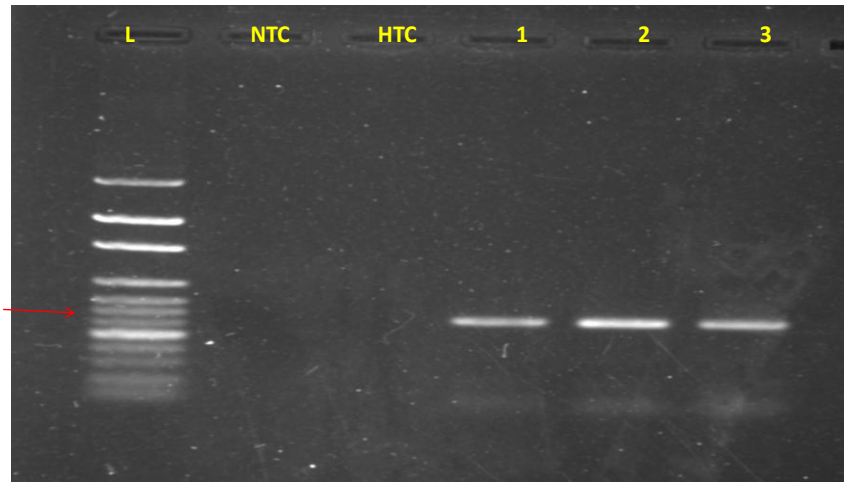
Prmimers ID	Amplicon size (bp)
BTC-CTV-1	500
BTC-CTV-1	70
BTC-CTV-1	250
BTC-CTV-1	300
BTC-CTV-1	300
BTC-CTV-1	500
BTC-CTV-1	400
BTC-CTV-1	480
BTC-CTV-1	500
BTC-CTV-1	480

CTV symptomatic leaf sample from citrus

CTV symptomatic leaf sample from sweet orange

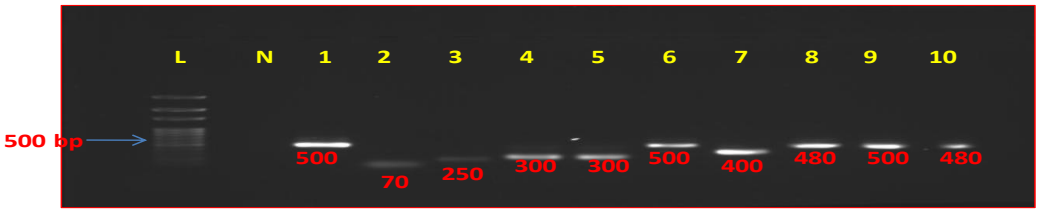
CTV symptomatic leaf sample from Lemon

Plate. 1 Collection of symptomatic leaf sample from citrus spp.



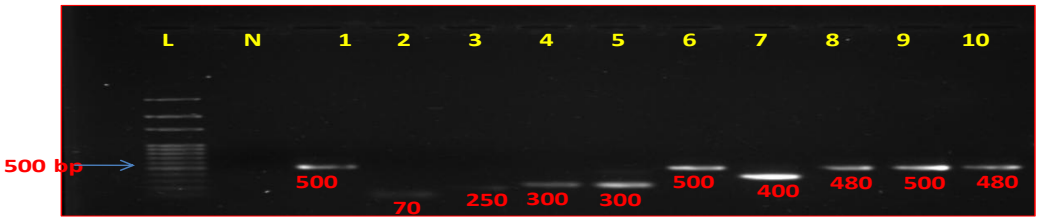
L- 100 bp Ladder, NTC- Non template control, HTC- Healthy control
1- orange c-DNA , 2- sweet orange C-DNA, 3-lemon C-DNA

Plate.2.Confirmation of presence of CTV by using T-36 (coat protein gene specific) primer.



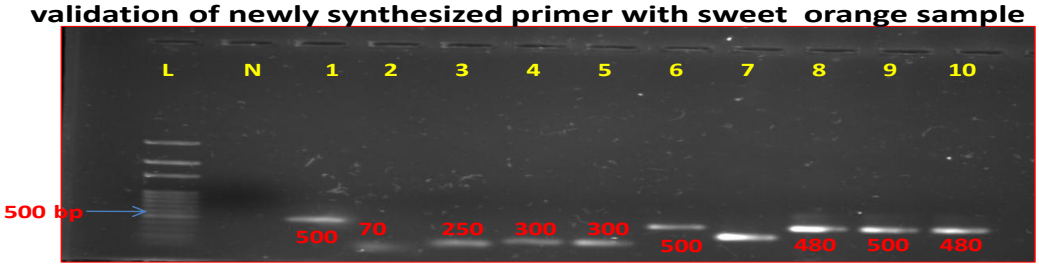
L-100 bp, N- -Ve control, 1-BTC-CTV1, 2-BTC-CTV2, 3-BTC-CTV4, 4-BTC-CTV5,
5-BTC-CTV6, 6-BTC-CTV7, 7-- BTC-CTV8, 8-BTC-CTV10, 9-BTC-CTV11, 10-BTC-CTV12

validation of newly synthesized primer with orange sample



L-100 bp, N- -Ve control, 1-BTC-CTV1, 2-BTC-CTV2, 3-BTC-CTV4, 4-BTC-CTV5,
5-BTC-CTV6, 6-BTC-CTV7, 7-- BTC-CTV8, 8-BTC-CTV10, 9-BTC-CTV11, 10-BTC-CTV12

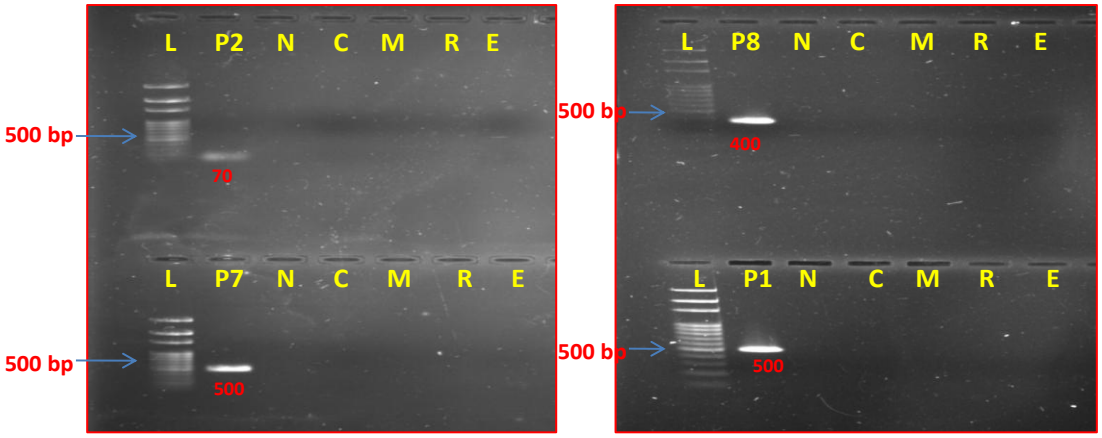
validation of newly synthesized primer with sweet orange sample



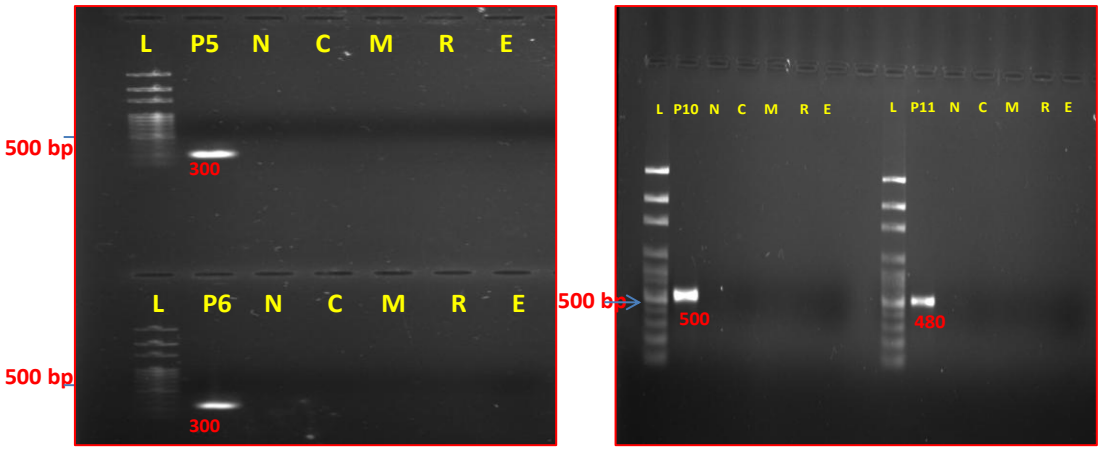
L-100 bp, N- -Ve control, 1-BTC-CTV1, 2-BTC-CTV2, 3-BTC-CTV4, 4-BTC-CTV5,
5-BTC-CTV6, 6-BTC-CTV7, 7-- BTC-CTV8, 8-BTC-CTV10, 9-BTC-CTV11, 10-BTC-CTV12

validation of newly synthesized primer with Lemon sample

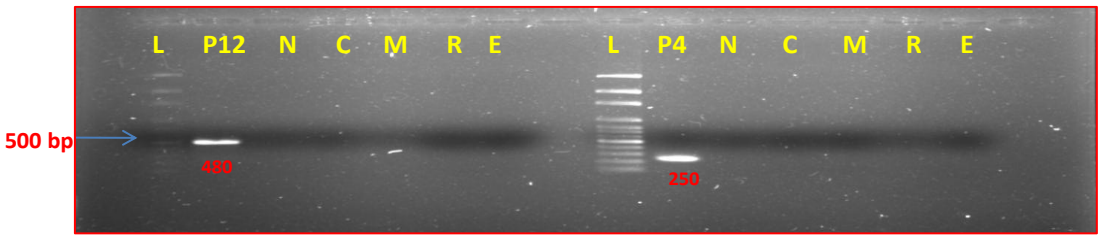
Plate.3. Detection of CTV by using new designed coat protein gene specific primers



L-100 bp, N- -Ve control, P2-BTC-CTV2 and c-DNA of CTV, P7-BTC-CTV7, P8-BTC-CTV8, P1-BTC-CTV1, C- Cranky leaf virus of citrus M- Mosaic Virus R-Ring spot virus E- Exocortis virus



L-100 bp, N- -Ve control, P5-BTC-CTV5 and c-DNA of CTV, P6-BTC-CTV6, P10-BTC-CTV10, P11-BTC-CTV11, C- Cranky leaf virus of citrus ,M- Mosaic Virus, R-Ring spot virus, E- Exocortis virus



L-100 bp, N- -Ve control, P12-BTC-CT12 and c-DNA of CTV, P4-BTC-CTV4 C- Cranky leaf virus of citrus M- Mosaic Virus R-Ring spot virus E- Exocortis virus

Plate.4. Validation of CTV CP gene specific primers with other major viruses of Citrus spp.

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