The construction of BCTV infectious clone to produce symptoms in the tomato plants

Habiba Naz[1], *Muhammad Azmat Ullah Khan[1], Abdul Qayyum Rao[2]

[1]Department of Biochemistry & Biotechnology, University of Gujrat, Pakistan

[2]Center of Excellence in Molecular Biology, University of the Punjab, Lahore Pakistan

Abstract

Curtoviruses is one of the important genus of *geminiviridae,* transmitted by the leafhopper *Circulifer tenellus.* Virus belong to *Curtoviruses* infect dicotyledonous plants and have circular single-strand DNA, a monopartite genome (2.9 kb). To characterize any virus at biological and molecular levels, producing an infectious clone of particular virus is essential. Traditional techniques for constructing infectious clones are laborious and time consuming due to many cloning and extraction steps required. We demonstrate a very effective and simple approach to produce an infectious clone of BCTV using Snapgene software, which significantly reduces all the cloning steps. The efficiency of this method was evaluated by confirming the infectivity of BCTV clones using agroinfection.

Introduction

Tomato (*Solanum lycopersicum L.*) is a major vegetable crop in Pakistan and a cost-effective and useful source of vitamin C for the human diet. Around twenty-five diverse viruses are reported in aging tomato crops worldwide [1]. The most important viruses that infect tomatoes naturally are *Tomato mosaic virus* (ToMV) [2], Tomato *yellow leaf curl virus* (TYLCV) [3], *Cucumber mosaic virus* (CMV) [4], *Beet curly top virus* (BCTV) [5], *Potato virus Y* (PVY) [6] and *Tobacco mosaic virus* (TMV) [7].

Geminiviridae family is the largest family of plant viruses. *Geminiviruses* are characterized by double icosahedral capsids that may have one or two single-stranded DNA genome(s). *Geminiviruses are* classified into nine genera on the roots of the insect vector, their genome structure and host plant range. The genera include *Grablovirus, Becurtovirus, Eragovirus, Begomovirus, Capulavirus, Mastrevirus, Topocuvirus, Curtovirus* [8]. When the *Geminivirus* attack on the crop, they cause changes in many plant pathways [9] that include: (a) the variations in the functions and structure of plasmodesmata [10], which are associated with defense mechanisms of host cell [11] (b) the interaction between various proteins involved in regulating development [12] (c) hyperplasia [13]. (d) Increase or decrease in the regulation of gene expression and (e) the interference in the pathway related to retinoblastoma [14].

Among these genera, *Curtoviruses* is one of the important genera of *geminiviridae,* the leafhopper Circulifer tenellus transmit them. *Curtoviruses* infect dicotyledonous plants and consist of a monopartite genome (2.9 kb). They encode V1 and V2 (coat protein) at virion - sense strand. Four

open reading frames (ORFs) exist on the complementary sense strand: C2, Rep, C4 and REn. The representative member of this genera is BCTV [15].

The coat protein (CP) encodes for viral capsid and regulate vector transmission. V2 also encoded protein involved in virus movement activity. Rep (AC1/C1) encode replication initiator protein which initiated the viral replication. C2 encode transcriptional activator protein (TrAP). C3 encodes REn (replication enhancer protein), which is very important for viral replication. The C4 in *curtovirus*es is still uncharacterized. The *curtoviruses* genomes also have a bidirectional promoted and highly conserved 5′ intergenic nanonucleotied (TAATATTAC) region which contain origin for rolling circle replication (RCR) [16].

Material and methods

Solanum lycopersicum Plant

Seeds of Tomato *(Solanum lycopersicum*) were grown in insect free chamber. 4-5 seeds were sown into small plastic pots contain soil and peat moss in 1:1. Seeds were grown into greenhouse with conditions of 25±3°C, 40% Relative humidity, and 8 h dark and 16 h light photoperiod. After germination, seedlings were transferred in new plastic pots (one seedling per pot). Plants with 4–5 fully expanded leaves (Three to four weeks old) were used for agro infiltration.

Construction of infectious clones

An infectious clone was synthesized using BCTV full length genome (gene bank accession number KT583749.1). Nonanucleotide region was split in both end. DNA fragments of 60 nucleotide were added at both ends to duplicate the nonanucleotide region in the BCTV genome.

Cloning into pCAMBIA1301

pCAMBIA1301 vector was used to clone the viral genome with duplicated nonanucleotide region. BCTV sequence was inserted at *SalI* site of vector. *SalI* restriction sites were also added at both end of BCTV sequences for competent insertion. Full length genome can separate with single enzyme *Sal1* digestion. Short fragment with nonanucleotide region can be separated by digesting with the enzyme *SalI* and *HindIII.* This infectious clone was synthesized by Synbio Technologies, USA.

Agroinfiltration

The construct BCTV was incorporated into *Agrobacterium tumefaciens* by electroporation. [17]. YEB medium was used to incubate Agrobacterium cells supplemented with 50 mg/L of rifampicin and 50 mg/L of kanamycin at 30 ◦C, 220 rpm for 48 h. single cell colonies from these incubated plates was selected and grown overnight in YEP broth with Kanamycin and Rifampicin. The fresh culture was prepared under same conditions to a final OD600 concentration of 0.5. after that culture was centrifuged at 8000× g for 3 min and pallet was resuspended in agroinfiltration buffer

(10 mM MES of pH 5.6; 10 mM $MgCl_2$; 150 µM acetosyringone) The suspended Agrobacterium cells were kept at room temperature in the dark for about 2 h. To check the infectivity of BCTV infectious clone on its host plant, 4–5 week old leaves of tomato was infiltrated in three replicates by using 1ml syringe without needle. Mock Agrobacterium without the construct was used to infiltrate control plant. Other tomato plants were infiltrated with BCTV in leaf near apex of lamina. The experiments was performed in three replicates. After inoculation, samples was observed and recorded after 7,14 and 21 days.

Complete genome extraction

After observing phenotypic results of agroinfiltration whole genome was extracted from infected leaves by using CTAB method. Wash infected leaves and keep it in -80 degree for 30 minuts. Then homogenized by using ice cold pester and mortal. Homogenized leaves were collected into 1.5 ml tube and CTAB solution added.

Restriction digestion analysis

Whole genome digested by using two different restriction enzymes. 2 ul of each enzyme was used to digest 2ul genome in v5 buffer.

Gel electrophoresis

Run the product of restriction digestion in 0.1% gel. Gel was prepared by using tris buffer, agarose and distilled water. 2ul of sample mixed with 1ul of dye and pour into wells of gel one by one. Gel electrophoresis performed for 30 mints at 80 volt and 400 amp. View gel by using gel doc.

Results

Growth of tomato plant

Tomato seed start germinating within 15 days under greenhouse controlled conditions. Germinated seedlings were transferred into new plastic pot maintained into insect fee chamber. After three to four weeks, plant have 4-5 fully extended leaves that can be used for agroinfiltration (Fig #1).

Construction of the infectious clone

The infectious clone was constructed by using Snapgene. The complete full-length genomic sequence of BCTV, having 2925 nucleotides, was downloaded into FASTA format from the NCBI website. The nonanucleotide sequence of BCTV split into two ends plays an important role in virus replication. To create duplicated nonanucleotide region in BCTV clone, short fragments of nucleotides are added at both ends. The first nonanucleotide region was present at position 54 and the second at position 2969. Then restriction site for *Sal1* 5'GATGAC 3' was added at both ends to make it compatible with ligation in pCAMBIA1301 at *Sal1* site. Using Snapgene restriction and insertion, cloning was performed at Sal1 site of pCAMBIA 1301 and insert single fragment of BCTV having duplicated nonanucleotide region. The size of final construct was 14881bp.

Complete BCTV genome is highlighted in blue, and the short fragment with second nonanucleotide region is highlighted in orange.

Confirmation of the infectious clone

Restriction digestion analysis was performed for confirmation of the infectious clone. Digestion was done by *sal1* restriction enzyme yielding 3 Kb fragment having duplicated nononucleatide region. Symptoms production in tomato leaves also confirm the efficiency of infectious clone.

Agroinfiltration in tomato

Tomato plants were infiltrated with the infectious clone of BCTV. In control plant so symptoms was produced while in other agroinfilterated pant start showing symptoms after 7 day. At 7 day plants symptoms was mild. At day 14 plant show much clear symptoms of BCTV. At day 21 plant show clear symptoms and growth of plant was disturbed (Fig #2.

At day 7: growth stunted

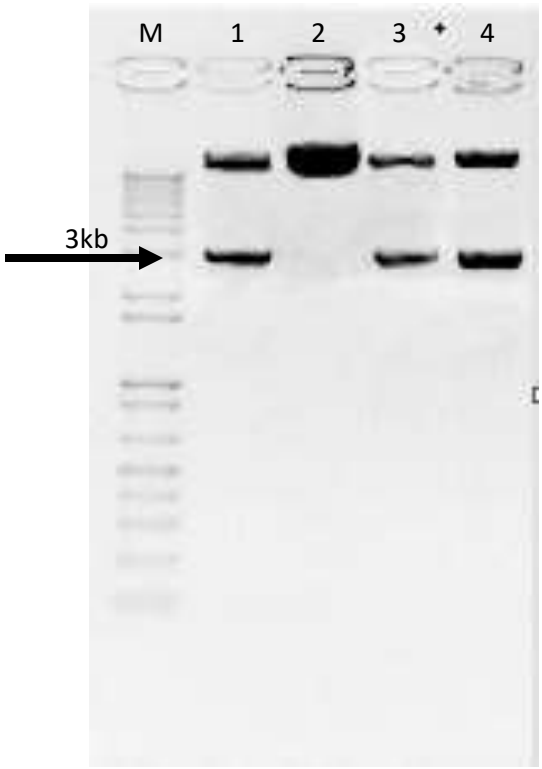
At day14: leaves become thicker and crisp and roll upwards

At day 21: petioles of the leaf roll downwards and turn in dull yellow with purple-colored veins

Fig#1: Agroinfiltration of tomato plants with BCTV (a) Control plant with no symptoms (b) plants Tomato plant with upward curlinf og leaves and vein thickening symptom

Restriction digestion product

CTAB isolated the whole genome form infected tomato leaves. Digestion of genome with *SalI* and *HindIII* of restriction enzyme yielding 3kb and 11 kb fragment. Fragment clearly shown on agarose gel under first well (fig #3).



Fig#2:Cofirmation of the infectious clone with restriction digestion product. By digesting with SalI and HindIII to produce ~3Kb fragment. Here M is 1KB ladder, lane 1 contain positive control, lane 2 shows negative control, whereas lane 3 &4 are the experimental samples

Discussion

Food security is one of the leading current global challenges due to rapidly increasing global population. But there are many threat for agriculture in environment, Plant-virus diseases are on top of the list. Virus infection causes huge crop loss by reducing plant productive life [18]. Most destructive plant viruses are the members of tospoviruses, begomoviruses, curtovirus and potyviruses. Plant viruses cause severe yield losses in varioust crops are belong to genus tospoviruses, tobamovirus, begomoviruses, potyviruses, cucumoviruses, allexivirus cutrovirus, carlavirus,tungro virus, babuvirus, badnavirus, and polerovirus[19].

BCTV is member of curtovirus. Previous studies have shown it causes vein swelling curly of leaves and discoloration of the plant. In previous study construction the infectious clone of Gemini virus performed by using different strategies to determine its effect on plant growth, development and fruit production. In 2015 blawid. R., et al., us gibson assembly to construct an infectious clone of tomato blistering mosaic virus (ToBMV) [20]. In 2016, Omid Eini et al., constructed a BCTIV head-to-tail partial dimmer.a fragment of 1028 bp was seoerated from pGem-1.0BCTIV-Kaf by digesting with *Hind*III/*Eco*RI enzymes. The released fragment was sub-cloned into corresponding sites of pBin20, a binary vector [21]. DNA was isolated to extract the viral genome in another study total plant. viral genome's was amplified, cloned and multiplied to construct complete

infectious clone. At last transformed into Agrobacterium tumefaciens and transfected into host plants to construct an infectious clone for Begomovirus.

In 2019 Ferro. M., et al., obtained an infectious clone of the geminivirus BGMV (Bean golden mosaic virus) by performing PCR and Gibson Assembly (GA). Sever symptoms of yellow mosaic virus appeared on *Phaseolus vulgaris* seedlings after 15 days of agroinoculation [22]. In 2021 Kikyo Watanabe et al., extract and amplify BCTV genome. Restriction enzyme zindIII/SalI and SalI/SacI digest amplified fragments one by one and cloned collected into pUC18 plasmid digested with HindIII/SacI to construct an infectious plasmid clone that have two IRs, pUC18-BCTV. They sequence the infectious clone using the appropriate primer and then clone into *Agrobacterium* binary vector pCAMBIA-2300 to construct pCAMBIA-BCTV1 infectious clone.[23]

Synthesis of RNA or DNA infectious clones of plant viruses has been renovated to a standard laboratory technique that provides a tremendous tool for researching virus-host interactions and viral gene functions. In the present study, we construct the infectious clone of BCTV using Snapgene software to determine its effect on plant growth, development and fruit production. Infectious clone have the complete genome of BCTV and can easily replicate into plant. After agroinfiltration phenotypic results of BCTV were observed after 7, 14 and 21 days. The infectious clone construction method and agroinfiltration techniques greatly impact BCTV expression. Tomato plant, which was infiltrated with mock agrobacterium, showed no symptoms of BCTV. But other tomato plants start showing symptoms after 7 days and symptoms become wore worst day by day. After 21 days growth of leaves was almost completely in control of BCTV. Other studies also prove that symptoms start producing after seven days and increase daily. The following study helps us to observe the effect of BCTV on plant growth and development.

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