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論　文
Articles

Infectivity Analysis of the Cloned Component of a Monopartite Begomovirus-Betasatellite Complex Causing Tomato Leaf Curl Disease in Pakistan

By

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Summary：*Cotton leaf curl Rajasthan virus* (CLCuRaV), a monopartite *begomovirus* causing devastating losses of cotton crop in India, was isolated from tomato (*Solanum lycopersicum*) along with DNA satellite (CLCuRaB^{tomato}) and characterized in Faisalabad, Pakistan. Infectious clones of CLCuRaV^{tomato} and CLCuRaB^{tomato} were produced and transformed into *Agrobacterium* strain GV3101. A partial tandem repeat construct of CLCuRaV^{tomato} was inoculated to *Nicotiana benthamiana* and tomato plants alone and/or with CLCuRaB^{tomato}. CLCuRaV^{tomato} alone produced very mild symptoms in *N. benthamiana* plants, whereas tomato plants showed no symptoms by inoculation with CLCuRaV^{tomato} in the absence of CLCuRaB^{tomato}. In *N. benthamiana* plants 100% infection followed by severe symptoms was observed while in tomato plants 70–80% infection with mild symptoms was observed on co-inoculation with CLCuRaV^{tomato} and CLCuRaB^{tomato}. Symptoms severity of CLCuRaV^{tomato} increased in the presence of CLCuRaB^{tomato} in *N. benthamiana* and tomato plants. Here we demonstrated the first experimental pathogenicity analysis of a cotton virus isolated from tomato plants both in *N. benthamiana* and tomato plants.

Key words：Agroinoculation, begomovirus, pathogenicity, satellite, tomato

Introduction

Members of the family *Geminiviridae* are plant-infecting viruses with genomes consisting of circular, single-stranded DNA (ssDNA) and geminate particles (Stanley *et al.,* 2005). They have been grouped into four genera (*Begomovirus, Curtovirus, Mastrevirus* and *Topocuvirus*) based on genome organization, host range and insect vector (Hanley-Bowdoin *et al.,* 1999 ; Stanley *et al.,* 2005). Most of the members of the genus *Begomovirus* are transmitted by whitefly (*Bemisia tabaci* : Gennadius), and they infect dicotyledonous plant species (Stanley *et al.,* 2005). Some *begomoviruses* possess a genome of two components, designated as DNA A and DNA B, of approx. 2.7 kb each. DNA A encodes replication-associated protein (Rep), transactivator protein (TrAP), replication enhancer protein (REn) and AC4 on complementary sense and coat protein (CP) and pre-coat protein on virion sense (Hanley-Bowdoin *et al.,* 1999). DNA B encodes a nuclear shuttle protein (NSP) and a movement protein (MP) both of which are essential in efficient systemic spread and symptom expression (Hanley-Bowdoin *et al.,* 1999). Many *begomoviruses* have a single genomic component resembling DNA A, which is capable of autonomous replication and movement within the plant (Chakraborty *et al.,* 2003 ; Dry *et al.,* 1993 ; Navot *et al.*, 1991 ; Kheyr-Pour *et al.,* 1991).

Tomato leaf curl disease (ToLCD) is a serious problem throughout the warmer parts of the world (Czosnek and Laterrot, 1997). On the Indian subcontinent, the disease is caused by a diversity of species of ssDNA viruses in the genus *Begomovirus* (Stanley *et al.,* 2004). *Tomato leaf curl New Delhi virus* (ToLCNDV) was first identified as causing the disease on the sub-continent in the 1990s, and has a typical bipartite *begomovirus* genome (Padidam *et al.,* 1995). In recent years, a diverse range of monopartite *begomoviruses* (viruses which lack the DNA B

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component) have been shown to cause ToLCD (Briddon *et al*., 2001). The majority of these viruses are associated with DNA satellite known as betasatellite (formerly known as DNAβ). Betasatellite is symptom-modulating, and occurs only in the Old World (Briddon and Stanley 2006 ; Jose and Usha 2003 ; Saunders *et al.,* 2000 ; Saunders *et al.,* 2003). Betasatellite depends on its helper virus for replication and encapsidation, and its presence is essential for symptom expression (Briddon and Stanley 2006).

Here in this paper we have discussed the infectivity analysis of a monopartite-begomovirus betasatellite complex (a cotton virus isolated from tomato) in a model host, *N. benthamiana*, and the natural host, tomato plants, by *Agrobacterium*-mediated inoculation.

Materials and Methods

In 2007, tomato (*Solanum lycopersicum*) plants exhibiting yellowing, leaf curling, vein thickening and mosaic symptoms were collected from Faisalabad, Pakistan. We isolated *Cotton leaf curl Rajasthan virus* (CLCuRaV-[PK : SA1 : 05]) associated with DNA satellite CLCuRaB-[PK : FSD : 05] (clone and accession no. SA1 ; AM501481 and Sβ2 ; AM490309 respectively) from these symptomatic tomato plants (Shahid *et al.,* 2007).

Construction of infectious clone of CLCuRaV^{tomato}

For infectivity analysis using *Agrobacterium*-mediated inoculation technique, partial repeat constructs of CLCuRaV^{tomato} and CLCuRaB^{tomato} were produced by general method. pGreen0029 binary vector was digested with *Mlu*1 and *Pst*1. Subsequently an approx. 656 bp (containing hair pin) fragment of CLCuRaV^{tomato} already cloned in PTZ257R/T was released and ligated to produce pGSA1-0.4 (binary vector and partial part) (Hellens *et al.,* 2000). In the second step, full-length CLCuRaV^{tomato} insert was released by *Mlu*1 digestion and ligated into pGSA1-0.4 already linearized and treated with Calf Intestine Alkaline Phosphate (CIAP) to produce pGSA1-1.4 (a partial repeat construct) (Fig. 1).

Production of partial repeat constructs of CLCuRaB^{tomato}

Partial repeat construct of CLCuRaB^{tomato} was produced by inserting full length Sβ2 at *Kpn*1 restriction site in pGSβ2-0.6. pGSβ2-0.6 was produced by ligating approx. 519 bp partial fragment of CLCuRaB^{tomato} at *Kpn*1 and *Sal*I into the pGreen0029 binary vector already digested with *Kpn*1 and *Sal*I restriction enzymes (Fig. 1). The partial repeat constructs were finally transferred into *A. tumefaciens* strain GV3101 by electroporation.

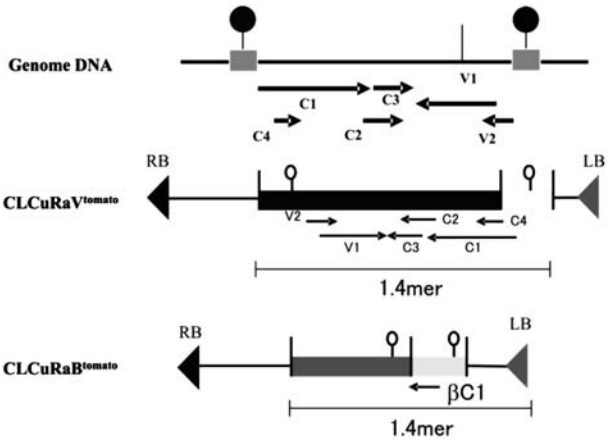

Fig. 1 pGSA1-1.4 partial repeat constructs of CLCuRaV^{tomato} (begomovirus) and pGSβ2-1.4 dimeric form of CLCuRaB^{tomato} (DNA satellite)

Transformation of infectious construct into *Agrobacterium*

Both of the constructs (CLCuRaV^{tomato} and CLCuRaB^{tomato}) were individually introduced into *A. tumefaciens*. Two microliters of the plasmid were mixed with electrocompetent *A. tumefaciens* cells on ice and transferred to a chilled electroporation cuvette. Electroporator (BTX Harvard Apparatus, Holliston, USA) was set at 1.44 kV. The cuvette was inserted into the electric shock chamber. After the electro shock, LB liquid medium was added to the cells in eppendorf tube and incubated at 28℃ for 2hrs in a shaker. Electroporated cells were spread on LB medium with appropriate antibiotics Kanamycin (30 ug/mL), Rifampicin (50 ug/mL) and Tetracycline (12 ug/mL) wrapped with aluminum foil and incubated at 28℃ for 48hrs. *A. tumefaciens* cells were grown at 28℃ for about 48hrs, and their cells were pelleted, washed with 10 mM $MgCl_2$ and resuspended in 1/10 the initial volume of 10 mM $MgCl_2$ or till the OD600 reached 1.0. *Agrobacterium* cultures were prepared and inoculated to plants as described previously (Hussain *et al.,* 2007).

Agroinoculation of *N. benthamiana* and tomato seedlings

N. benthamiana and tomato seedlings were agro-infiltrated with CLCuRaV^{tomato} alone and/or together with CLCuRaB^{tomato} in three independent experiments. In three replicates twelve plants from *N. benthamiana* and tomato at the 4th to 6th leaf stage were agroinoculated. The bacterial preparation was injected into tomato and *N. benthamiana* seedlings by slightly puncturing the leaf with a clean pin and the bacterial suspension was pressurized into the leaf stab using a disposable sterile syringe (2-mL) with

26G1/2 needle for tomato plants and without needle to *N. benthamiana* palnts. Only binary vector was agroinoculated as a mock control. Non-inoculated seedlings were also included as healthy control. The agro-infiltrated plants were monitored for symptoms appearance in insect-free and secured growth rooms at 28℃, daily cycle of 16hrs light and 8hrs dark for four weeks.

Polymerase chain reaction analysis

Detection of begomovirus components were done by PCR using *begomovirus* specific primers (Shahid *et al*., 2007) BegF ACGCGTGCCGTGCTGCTGCCCCCATTGTCC and BegR ACGCGTATGGGCTGYCGAAGTTSAGAC for $CLCuRaV^{tomato}$ and β01 GGTACCACTACGCTACGCAGCAGCC β02GGTACCTACCCTCCCAGGGGTACAC for $CLCuRaB^{tomato}$. The thermal cycler was programmed for a preheat treatment of 94℃ for 5 min followed by 35 cycles of 94℃ for 1min, 48℃ to 52℃ for 1min and 72℃ for varying times (dependent upon the length of fragment to be amplified ; typically 1 min per 1000 nucleotides to be amplified), followed by a final incubation of 10 min at 72℃ and the machine was set to hold at 4℃ until the samples were removed.

DNA extraction and rolling circle amplification analysis

Total DNA was extracted from *N. benthamiana*, tomato and mock inoculated plants, as described previously (Doyle and Doyle 1987). To confirm the presence of viral components in *N. benthamiana* and tomato hosts, the genomic DNA of agroinoculated *N. benthamiana* and tomato plants was used as a template to $CLCuRaV^{tomato}$ and $CLCuRaB^{tomato}$ molecule using rolling circle amplification (RCA) technology (Nahid *et al.,* 2008). For amplification of circular DNA molecules by RCA a reaction mixture of 20 μL containing 100 to 200 ng genomic DNA of infected plant samples, 50 μM random hexamer primers, 2 μL 10X Φ29 DNA polymerase reaction buffer (330 mM Tris-acetate [pH 7.9]), 100 mM magnesium acetate, 660 mM potassium acetate, 1% (v/v) Tween 20, 10 mM DTT) was prepared and incubated at 94℃ for 3 min to denature double stranded DNA. The mixture was cooled to room temperature, mixed with 1 mM dNTPs, 5–7 units of Φ29 DNA polymerase and 0.02 unit of pyrophosphatase to eliminate inhibitory accumulation of pyrophosphate and incubated at 30℃ for 18 to 20hrs. The following day Φ29 DNA polymerase was inactivated at 65℃ for 10 min.

Southern hybridization analysis

Genomic DNA 10 μg per well was loaded on a 1% (w/v) agarose gel and run at 40 V (Biorad PowerPacTM) in TBE buffer for 4 to 5hrs. Gel was stained with 0.5 μg/mL ethidium bromide and DNA image was obtained under UV light in gel documentation apparatus (Eagle Eye-Stratagene). After electrophoresis the gel was treated with depurination solution (0.25M HCl) for 15 min, denaturation solution (1.5M NaCl and 0.5M NaOH) for 30 min and neutralization solution (1M Tris [pH 7.4], 1.5M NaCl] for 30 min. The gel was rinsed briefly with distilled water between treatments and shaken moderately on platform shaker during each treatment. DNA in the gel was transferred to a nylon membrane (Hybond-Amersham) in 10X SSC and/or sometimes in 5X SSC (1.5M NaCl and 150 mM sodium citrate) by capillary action. The DNA on the nylon membrane was crosslinked by UV irradiation (CL-1000, UVP) at 120 mJ/cm^2 energy. The membrane was then rinsed in a solution containing 0.1X SSC, 0.5% (w/v) SDS at 65℃ for 45 min to remove residual agarose.

Before hybridization the membrane was treated with 0.2 mL/cm^2 pre-hybridization solution (6X SSC, 5X Denhardt's solution [0.1% (w/v) each of bovine serum albumin, 0.5% (w/v) Ficoll (Mol. Wt. ~400,000) and PVP (Mol. Wt. ~40,000)], 50% (v/v) SDS) and 5 mg/mL sheared and denatured salmon sperm DNA at 42℃ for 2–4 hrs in a hybridizer (Hybaid, Midi Dual 14), to block non-specific binding sites. DNA probes were prepared using a Biotin DecaLabel DNA Labelling kit (Fermentas) according to the manufacturer's instructions. Briefly, in a 1.5 mL microcentrifuge tube a 44 μL reaction mixture was prepared by adding 50–200 ng DNA template (usually purified PCR product), 10 μL decanucleotide in 5X reaction buffer and nuclease free water. The reaction mixture was vortexed briefly, centrifuged briefly in a microfuge to collect the contents at the bottom of the tube and incubated in a boiling water bath for 5 to 10 min. After incubation the tube was cooled on ice, briefly microfuged and the contents of the tube mixed with 5 μL biotin labelling mixture and 1 μL Klenow fragment exo- (5units) and incubated at 37℃ for 1 to 20 hrs. Reaction was stopped by adding 1 μL 0.5M EDTA [pH 8.0]. To prepare hybridization solution, the biotin labelled probe was denatured at 100℃ for 5 min, chilled on ice and mixed with pre-hybridization solution (25–100 ng/mL).

After 2 to 4hrs treatment, the pre-hybridization solution was discarded and the hybridization solution was added to the membrane (60 μL/cm^2) and incubated overnight in a hybridizer at 42℃. The following day the membrane was washed twice with 2X SSC/0.1% (w/v) SDS at room temperature for 10 min. The membrane was washed with 0.1X SSC/0.1% (w/v) SDS twice at 65℃ for 20 min. To detect the biotin-labelled DNA the membrane was washed in 30 mL Blocking/Washing Buffer (provided by the manufacturer) at room temperature. After 5 min the membrane was treated with 30 mL

Blocking Solution for 30 min to block non- specific binding sites on the membrane. Streptavidin-AP conjugate was diluted in 20 mL Blocking Solution and the membrane was incubated in it for 30 min. The membrane was washed twice in 60 mL Blocking/Washing buffer for 15 min and incubated with 20 mL Detection Buffer for 10 min. Finally the membrane was treated with 10 mL freshly prepared substrate solution at room temperature in the dark until blue-purple precipitate became visible. To stop the reaction, the substrate solution was discarded and the membrane was rinsed with water. The blot was immediately photographed and the membrane was then air dried and stored.

Results and Discussion

Symptom expression

N. benthamiana seedlings agroinoculated with CLCuRaV^{tomato} alone produced very mild curling and stunted growth in only three out of thirty six *N. benthamiana* plants (Fig. 2 B), while none of the tomato seedlings was found to be infected within 21 days of post inoculation (dpi) (Fig. 2). *N. benthamiana* plants agroinoculated with CLCuRaV^{tomato} and CLCuRaB^{tomato} showed severe upward leaf curling, reduction in size, cupping and stunted growth in 14 dpi (Fig. 2 C). Tomato plants exhibited some yellowing and mild curling at 28 dpi with CLCuRaV^{tomato} and CLCuRaB^{tomato} (Fig. 2 F). Mock inoculated plants showed no symptoms (Fig. 1 panel A ; *N. benthamiana* and D ; tomato). One hundred percent infection was observed in *N. benthamiana* and approx. 70 to 80% tomato plants were symptomatic when inoculated with CLCuRaV^{tomato} and CLCuRaB^{tomato} (Table 1).

Detection of viral components by RCA in *N. benthamiana* and tomato plants

The RCA product was digested with the unique restriction sites to confirm the presence of CLCuRaV^{tomato} and CLCuRaB^{tomato} components. RCA analysis showed that the viral progeny of CLCuRaV^{tomato} and CLCuRaB^{tomato} were moved systemically in all *N. benthamiana* and only in one third of tomato plants. The agroinoculated *N. benthamiana* plants with only CLCuRaV^{tomato} cause 8% of infection but no detection was observed in tomato plants (Fig. 3 upper panel).

Detection of viral components by PCR in *N. benthamiana* and tomato plants

Specific *begomovirus* primers were used for DNA amplification of CLCuRaV^{tomato} and CLCuRaB^{tomato} from *N. benthamiana* and tomato plants. The PCR amplification was observed in the same plants that was already confirmed by RCA (Fig. 3 lower panel).

Virus component detection by Southern hybridization

To detect the CLCuRaV^{tomato} at low level threshold of RCA and PCR, southern hybridization was also carried out for final confirmation of viral DNA in both *N. benthamiana*

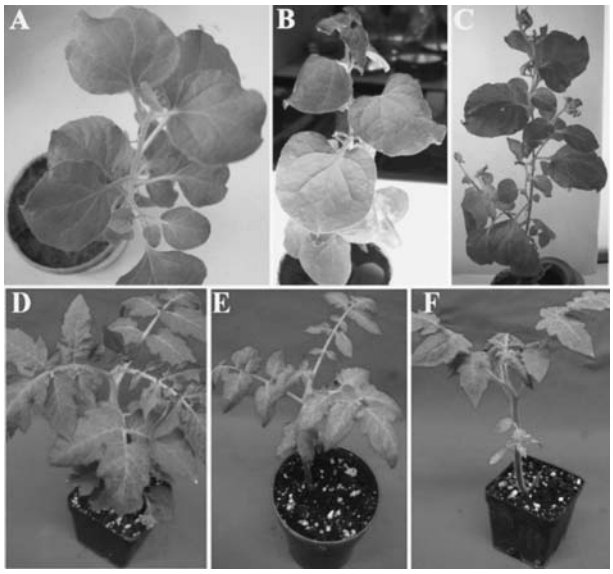

Fig. 2 Symptoms exhibited by *N. benthamiana* and tomato plants infected with CLCuRaV^{tomato} and CLCuRaB^{tomato} by *Agrobacterium*-mediated inoculation. *N. benthamiana* and tomato inoculated with CLCuRaV^{tomato} (B and E), CLCuRaV^{tomato}+CLCuRaB^{tomato} (C and F). Mock inoculated *N. benthamiana* (A) and tomato (D) plants are shown for comparison. Close-up photographs are shown to highlight the symptoms.

Table 1 Infectivity of *Cotton leaf curl Rajasthan* virus associated with betasatellite by *Agrobacterium*-mediated inoculation.

Inoculum	Plant	Infectivity (no. of plants infected/no. of plants inoculated)
CLCuRaV	*N. benthamiana*	0/32
CLCuRaV+CLCuMB		30/32
CLCuRaV	Tomato	0/32
CLCuRaV+CLCuMB		25/32

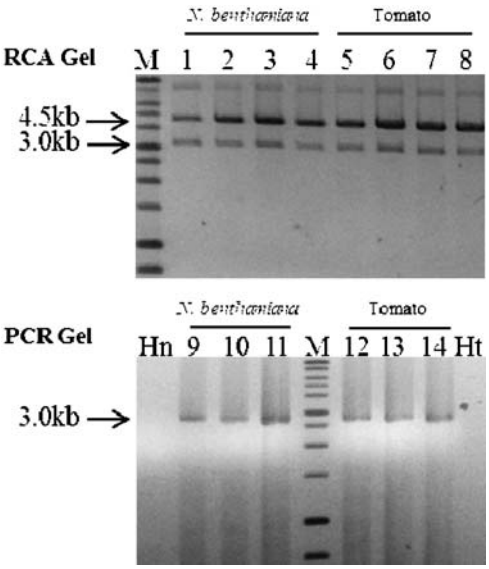

Fig. 3 Detection of viral components by RCA. RCA analysis of *N. benthamiana* and tomato plants agroinoculated with CLCuRaV^{tomato}/CLCuRaB^{tomato}. *N. benthamiana* (lane 1-4) and tomato (lane 5-8). Approx. 3.0 kb band shows full length helper virus. ~4.5 kb bands show dimeric form (upper gel). Detection of viral components by PCR. PCR assay of both *N. benthamiana* and tomato plants agroinoculated with CLCuRaV^{tomato}+CLCuRaB^{tomato}. *N. benthamiana* (lane 9-11) and tomato (lane 12-14). ~3.0 kb PCR band show full length amplification (lower gel). Hn mock inoculated (*N. benthamiana*) and Ht (tomato) M=Ikb marker was used in both assays.

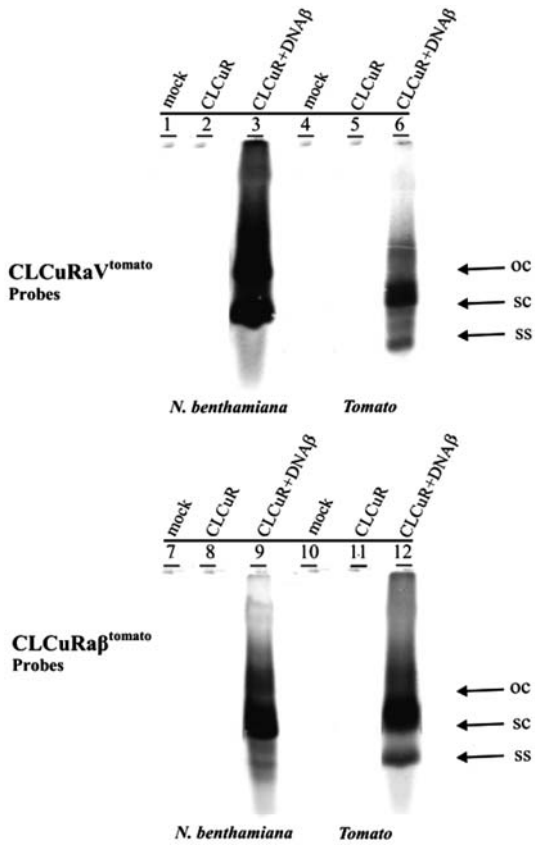

Fig. 4 Detection of viral component by southern hybridization. Blots were probed with fragments of (a) CLCuRaV^{tomato} begomovirus and (b) CLCuRaB^{tomato} DNA satellite. DNA samples (7 µg) were isolated from *N. benthamiana* and tomato plants agroinoculated with CLCuRaV^{tomato} (lane 2, 5, 8 and 11), CLCuRaV^{tomato}+CLCuRaB^{tomato} (lane 3, 6, 9 and 12). The sample in (lane 1, 4, 7 and 10) from both panels originated from a mock-inoculated plant (*N. benthamiana* and tomato, respectively).

and tomato plants. Southern blot analysis suggested that CLCuRaV^{tomato} can help in the replication and movement of CLCuRaB^{tomato} in *N. benthamiana* and tomato plants when probed with coat protein (CP) and βC1 of CLCuRaV^{tomato} CLCuRaB^{tomato}, respectively were labeled with digoxigenin-dUTP using the manufacturer's instructions (Roche Biomedical, Burlington, NC) (Fig. 4 upper panel probed with CLCuRaV^{cotton}, lower panel proved with CLCuRaB^{cotton}).

The agroinoculation data suggested to us that CLCuRaV^{tomato} is only a monopartite begomovirus responsible for severe leaf curl disease of tomato plants in Faisalabad, Pakistan. Shahnawaz-ul-Rehman *et al.,* (2009) have shown previously that *cotton leaf curl Rajasthan virus* (CLCuRaV^{cotton}) isolated from cotton plant alone cannot move systemically, and it requires DNA satellite to cause attenuated symptoms in *N. benthamiana* plants. The sequence comparison of CLCuRaV^{tomato} (accession no. AM490309 ; isolated from tomato) and CLCuRaV^{cotton} (EU365616 isolated from cotton ; *Gossypium davidsonii*) showed that both are 92% identical with each other. Also, it might be possible that CLCuRaV^{cotton} is missing some sequences that are required for systemic movement compared to CLCuRaV^{tomato}. Also comparison of CLCuRaB^{tomato} (AM490309) with other betasatellite CLCuB^{Mul}, CLCuB^{Bur} and ChLCB (FJ607041, EU384595 and FJ515274, respectively) used in the previous study showed only 79.8%, 63.2% and 37.9% sequence similarity with CLCuRaB^{tomato} (Shahnawaz-ul-Rehman *et al.,* 2009). Betasatellite is known to be required for induction of disease symptoms in several host-virus combinations (Briddon *et al.,* 2001 ; Jose and Usha 2003 ; Saunders *et al.,* 2003 ; Zhou *et al.,* 2003). This study further proved the pathogenicity of CuRaB^{tomato} betasatellite. The novelty of our work is to prove the pathogenecity of CLCuRaV^{tomato} and CLCuRaB^{tomato} isolated from tomato but not from cotton fields (that is the natural field host for this virus). Possibly, the host virus interaction plays a key role for the adaptation of this begomovirus-betasatellite complex in tomato crops. Also this is the first demonstration of

Koch's postulates using $CLCuRaV^{tomato}$ and $CLCuRaB^{tomato}$ by agroinoculation in tomato plants. The adaptation of this virus into tomato plants is a big future challenge for tomato production in Pakistan. The problem should be resolved in the near future ; otherwise it could be a big problem for tomato crops throughout the world.

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パキスタンに発生したトマト巻葉病を引き起こす単一ゲノム性 *Begomovirus* および β サテライトの感染性クローンによる感染性解析

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要約：インドのワタに壊滅的な被害をもたらす単一ゲノム性 *Begomovirus* 属ウイルスである *Cotton leaf curl Rajasthan virus*（CLCuRaV）がパキスタンの Faisalabad で栽培されるトマト（*Solanum lycopersicum*）から，DNA サテライト［CLCuRaB-(PK : Sβ2 : 07)］とともに分離され，CLCuRaV-(PK : SA1 : 07）と同定された。両者の感染性クローン（CLCuRaV^{tomato} および CLCuRaB^{tomato}）を構築し，*Agrobacterium* GV3101 系統に形質転換した。CLCuRaV^{tomato} を *Nicotiana benthamiana* とトマトに，単独で，あるいは，CLCuRaB^{tomato} と共接種したところ，3 反復おこなった CLCuRaV^{tomato} 単独接種実験では，*N. benthamiana* 36 株中 3 株のみで非常に軽微な病徴を呈し，トマトには感染が認められなかった。しかし，サテライト DNA 感染性クローン CLCuRaB^{tomato} との共接種によると，*N. benthamiana* では 100% の感染が認められ激しい病徴を呈し，トマトでは穏やかな病徴ではあるが 70–80% の感染が認められた。CLCuRaB^{tomato} の存在により CLCuRaV^{tomato} の病徴が *N. benthamiana* およびトマトにおいて激化した。これにより，トマトから分離したワタのウイルスである CLCuRaV^{tomato} の実験用宿主 *N. benthamiana* と野外宿主トマトにおける感染性を実験的にはじめて確認した。

キーワード：アグロ接種，Begomovirus，病原性，サテライト，トマト

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