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Cotton Leaf Curl Rajasthan Virus Infecting Tomato in Pakistan

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Abstract: Tomato plants showing phenotypically symptoms of tomato leaf curl disease (ToLCD) were collected in Faisalabad, Pakistan. These exhibited a severe downward leaf curling with enation on the lower side of the leaf. In order to identify the begomovirus components associated with the disease phenotypes, DNA extracted from them was screened by PCR using specific primers for ToLCNDV DNA A and DNA B and a universal beta satellite primer pair (designed to detect all beta satellite). Tomato sample was found positive for ToLCNDV DNA A and beta satellite. The fragments amplified were cloned and sequenced. The begomovirus sequence obtained showed the highest levels of sequence identity (99%) to cotton leaf curl Rajasthan virus (CLCuRV), a virus previously identified in cotton showing symptoms of cotton leaf curl disease (CLCuD). The sequence of beta satellite showed 99% identity to the beta satellite associated with CLCuD. This is the first time CLCuRV has been identified in tomato and indicates that this host can serve as a reservoir for the agent causing CLCuD. Partial repeat constructs for *Agrobacterium*-mediated inoculation have been produced to show infectivity of these clones (to fulfill Koch's postulates), for studying their host range and potential threat to crops.

Keywords: begomovirus, geminivirus, whitefly, tomato disease, beta satellite, CLCuRV in tomato, CLCuD

Introduction

Tomato leaf curl disease (ToLCD) is a serious problem throughout the warmer parts of the world (Czosnek and Laterrot, 1997). In the Indian subcontinent, the disease is caused by whitefly-transmitted Gemini viruses, a diverse range of single-stranded DNA viruses of the genus *Begomovirus* (family Geminiviridae) as reported by Stanley *et al*. (2004). The virus associated with ToLCD, first identified in the sub-continent in the 1990s, was tomato leaf curl New Delhi virus (ToLCNDV), a typical bipartite begomovirus; having two genomic components, designated DNA A and DNA B (Padidam *et al*., 1995). In recent years, a diverse range of monopartite begomoviruses (viruses which lack the DNA B component) have been shown to cause ToLCD. The majority of these viruses are associated with beta satellite, a recently identified group of symptom-modulating, single stranded DNA satellites that occur only in the Old World (Briddon and Stanley, 2006).

Cotton leaf curl disease (CLCuD) sporadically affects, in addition to cotton, a number of crop species including radish, papaya (Chowda Reddy *et al.*, 2005) and chili peppers (Hussain *et al.*, 2003; Mansoor *et al.*, 2000; Padidam *et al*., 1995). In most cases, the viruses causing the disease have been poorly characterised, although in each case the presence of the CLCuD beta satellite was shown. These infections of non-malvaceous hosts likely occur due to a high inoculum pressure. Faisalabad is an area situated in the centre of the cotton-growing belt of the Punjab province, Pakistan and during the summer season, few other crops are in the field. It is likely, therefore, that during this period, the whitefly vector, carrying the components (begomovirus and beta satellite) causing CLCuD, are in abundance.

CLCuD is a major constraint to cotton production in Pakistan. The disease became an epidemic in Pakistan during the 1990s and continues to cause problems throughout the cotton growing areas of the country. The disease is caused by a newly identified class of begomovirus (family Geminiviridae). These viruses are monopartite but require a satellite molecule (betasatellite) to induce typical disease symptoms in the plant species from which they were isolated (Jose and Usha, 2003; Mansoor *et al.*, 2003; Saunders *et al.*, 2003; Zhou *et al.*, 2003; Briddon *et al.*, 2001). Beta satellite components are symptom-modulating, single stranded DNA satellites that require the helper begomovirus for replication, spread in plant tissues, and plant-to-plant transmitted by the whitefly vector of begomoviruses (*Bemisia tabaci*). A third molecule, alpha-satellite is also associated with the begomovirus-beta satellite complex. Alpha satellite components are satellite-like molecules which appear to play no part in the disease process.

The present research was carried out as the virus has been isolated from the tomato crop. It could be due to the host range of this virus (begomovirus) or due to the polyphagus nature of the vector (whitefly). Since tomato is one of the main

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crops of our country, attention is needed to characterize different viruses infecting this crop and furthermore, to find some solution of these problems.

Materials and Methods

Collection of samples. Tomato (*Solanum lycopersicum*) plants showing distinct symptoms of ToLCD, were collected from Faisalabad, Punjab Province, Pakistan. These plants exhibited an unusual severe "leaf curl" phenotype consisting of highly curled leaves, reduced in size (Fig. 1) with frond-like enations on the veins on the underside.

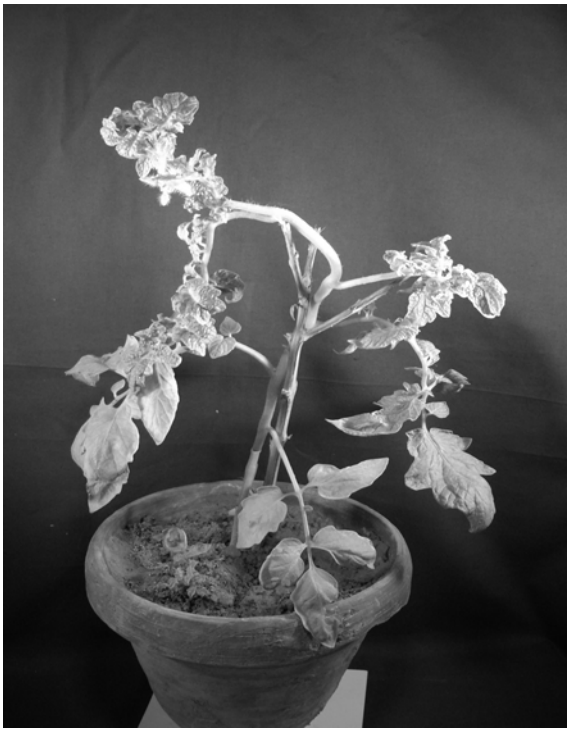

Fig. 1. Symptoms shown by the tomato plant.

Isolation of genomic DNA and analysis by PCR. Total nucleic acids were extracted using the CTAB method (Doyle and Doyle, 1987) whereas, initial analysis was done by PCR with universal primers (Briddon and Markham, 1994). Two primer pairs universal for begomoviruses, BF (5′-ACGCGTGCCGTGC TGCTGCCCCATTGTCC-3′)/ BR (5′-ACGCGT ATGGGCTG YCGAAGTTSAGACG-3′) and â01/ â02 (Briddon *et al.*, 2002), were used to amplify (PCR) the full-length begomovirus component and beta satellite, respectively, from the diseased plants (Fig. 2). Multiple components of DNA A and beta satellite were amplified from tomato plants. The reaction mixture consisted of template DNA (diluted) 5 ㎖, dNTPs (2 mM) 5 ㎖, PCR reaction buffer (10X) 5 ㎖, $MgCl_2$ (1.5 mM) 3 ㎖, primers (5 mM)

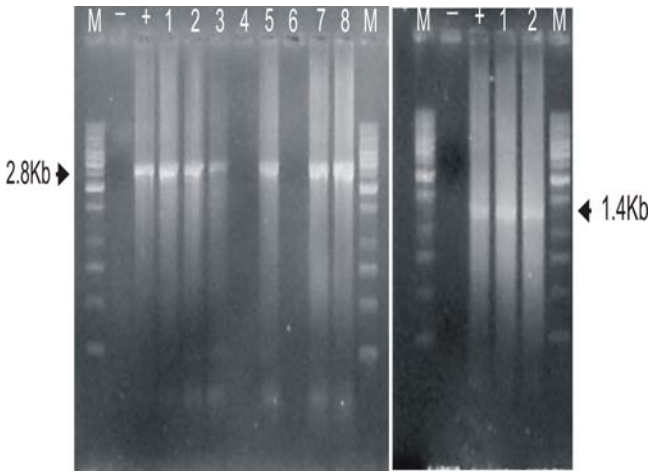

Fig. 2. Panel A shows PCR with DNA A specific primers. M shows marker, on both corners (-) negative and (+) positive control. Panel B shows PCR with betasatellite Primers. M shows marker, (-) negative and (+) positive control, respectively.

1 ㎖ of each (forward and reverse), Taq DNA polymerase 0.5 ㎖ and finally to make the volume 50 ㎖ nuclease free water was added. Denaturation, annealing and extention temperatures were set at 94, 50 and 72 °C, each for 1 min. 35 cycles were repeated for each PCR reaction. Amplified PCR products of expected sizes were cloned into pTZR/T cloning vector (Fermentas).

Sequencing. Plasmids were purified using a GeneJET Plasmid Miniprep Kit (Fermentas) and their sequences were determined commercially by Macrogen, South Korea. Multiple clones were obtained and one clone from each amplification (designated clone SA1 and Sâ2 for the begomovirus and beta satellite, respectively), was selected and sequenced in its entirety in both orientations.

Results and Discussion

Complete nucleotide sequences of clones SA1 and Sâ2 (available in the databases under accession numbers AM501481 and AM490309, respectively) were determined as 2753 and 1370 nucleotides, respectively. Sequences of begomoviruses and associated satellites used in the analyses were obtained from the sequence databases. Comparison of sequences showed the begomovirus (SA1) to be nearly identical to all isolates of cotton leaf curl Rajasthan virus (CLCuRV) available in the databases (over 99% nucleotide sequence identity). However, the sequence of SA1 also showed over 89% identity to a number of cotton leaf curl Multan virus (CLCuMV) isolates (86% to 94% identity for the isolates compared here). Phylogenetic comparisons show SA1 to cluster with the

CLCuRV isolates, being most closely related to an isolate originating from India (CLCuRV-[India: Abohar: 2003], AY795606). This indicates that the begomovirus identified to be the cause of severe ToLCD is an isolate of CLCuRV, for which we propose the descriptor CLCuRV-[Pakistan: Faisalabad: tomato: 2005]. Significantly, Abohar in India is only a short distance, across the border, from Faisalabad, and this suggests that CLCuRV may have spread into Pakistan from India. CLCuRV is one of at least 7 begomoviruses shown to be associated with cotton leaf curl disease (CLCuD) (Kirthi *et al.,* 2004; Mansoor *et al.,* 2003).

CLCuD is endemic to the majority of cotton cultivating areas of central southern Pakistan and western India. The sequence of Sb2 shows a typical arrangement of beta satellites, with a single open reading frame in the complementary sense (known as âC1; coordinates 508-155), a region of sequence rich in adenine (coordinates 719-1013), and a sequence motif highly conserved between all beta satellites, known as the satellite conserved region (SCR; coordinates 1265-14). The âC1 gene is predicted to encode a 118-amino-acid protein which shows more than 98% amino acid sequence identity to the âC1 products of other beta satellites isolated from cotton affected by CLCuD. The satellite does not contain recombinant SCR recently identified for the CLCuD beta satellite associated with the resistance breaking strain of CLCuD (Briddon *et al.,* 2001). Sâ2 is thus a typical CLCuD beta satellite of the type first identified in association with the disease epidemic that occurred during 1980s to 1990s (Briddon *et al.,* 2001). These infections of non-malvaceous hosts likely occur due to a high inoculum pressure.

Now, that the infectious clones of cotton leaf curl Rajasthan virus (CLCuRV) and its associated beta satellite have been produced, an analysis of infectivity will be conducted by *Agrobacterium*-mediated inoculation in the model plant *Nicotiana benthamiana* and in the natural host plant of the virus i.e. tomato.

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