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Mobilisation into cotton and spread of a recombinant cotton leaf curl disease satellite

Brief Report

I. Amin[1], **S. Mansoor**[1], **L. Amrao**[1], **M. Hussain**[1], **S. Irum**[1], **Y. Zafar**[1], **S. E. Bull**[2], and **R. W. Briddon**[1]

[1]National Institute for Biotechnology and Genetic Engineering (NIBGE), Faisalabad, Pakistan
[2]Department of Disease and Stress Biology, John Innes Centre, Norwich, U.K.

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Summary. Analysis of a DNA β satellite associated with a recently identified cotton leaf curl disease (CLCuD) strain indicated it to be recombinant, with most of the molecule originating from CLCuD DNA β but with some sequence from a satellite isolated from tomato. Analysis of both archival (pre 2001) and recent cotton samples, shows the recombinant satellite is confined to a small area but was not present in cotton prior to 2001. This indicates that the recombinant DNA β was recently mobilized into cotton, likely from tomato, and that recombination plays a role in the evolution of these satellites.

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Viruses of the family *Geminiviridae* are serious constraints to agricultural productivity in all tropical, sub-tropical and, increasingly, more temperate regions of the world. Most of the geminiviruses of significance to crop production are in the genus *Begomovirus*, being transmitted exclusively by the whitefly *Bemisia tabaci*, the majority of which have a bipartite genome structure [19]. Recently a new group of begomoviruses has been identified which consists of monopartite viruses that require a satellite molecule, DNA β, to symptomatically infect hosts [1, 16]. DNA β-requiring begomoviruses have been shown to be numerous but, apparently, confined to the Old World [5].

DNA β components are symptom-modulating, circular, single-stranded DNA satellites approximately 1350 nucleotides in size. They require the helper begomovirus for replication, movement in plants and insect transmission. Although they are infectious to plants, DNA β-requiring begomoviruses do not induce

typical disease symptoms in the absence of the satellite, and virus DNA levels are low. They encode an essential pathogenicity determinant that has a role in overcoming host plant defense [7, 15, 17].

Cotton leaf curl disease (CLCuD) is prevalent across Pakistan and western India. The disease is caused by a complex consisting of one or more begomoviruses (7 species have been identified so far) associated with a single DNA β satellite (CLCuD DNA β; [1, 11]). The present problems in cotton originated in 1985 in the vicinity of Multan (Pakistan) in highly susceptible cotton varieties and spread to almost all cotton growing regions, as well as into western India, where the disease is still epidemic [3].

Conventional breeding programs were successful in delivering CLCuD-resistant cotton varieties that were released to farmers during the 1990s [14]. Their cultivation was proving successful in alleviating losses to cotton production in CLCuD endemic areas and, for several years, the resistant varieties remained symptom free [13]. In 2001 the first symptoms of CLCuD in resistant varieties appeared in cotton crops in the vicinity of Burewala (district Vehari, Punjab province, Pakistan), indicating the appearance of a resistance-breaking strain of the disease [10]. In subsequent years the affected area has increased, signalling a probable second wave of the CLCuD epidemic.

In an effort to confirm the association of a DNA β with the new strain of CLCuD, leaf samples from cotton plants showing typical leaf curl symptoms, were collected in district Vehari (Pakistan), at the centre of the outbreak of CLCuD in apparently "resistant" cotton varieties. PCR-mediated amplification from nucleic acid extracts of these plants, using universal DNA β primers [2], yielded an approx. 1400-nucleotide product, indicative of the presence of a begomovirus-associated satellite DNA β (results not shown). The PCR product from a single plant was cloned, and two clones (CLCuDβ03-PK and CLCuDβ04-PK) were sequenced in their entirety (accession numbers AM084380 and AM084379, respectively).

Analysis of the sequence of CLCuDβ04-PK showed it to be a typical DNA β satellite; containing the single complementary-sense open reading frame conserved between all DNA βs (known as βC1; nucleotide coordinates 551 to 192, with the capacity to encode a 118-amino-acid product), an A-rich region (nucleotide coordinates 766 to 984 containing 58.9% A, compared to an overall 29.4% A content for the whole molecule) and a region of sequence which is well conserved between all DNA βs, referred to as the satellite conserved region (SCR; [4]). The SCR contains a predicted hairpin structure with the loop sequence TAATATTAC with similarity to the origin of virion-strand DNA replication of geminiviruses [1, 16]. The sequence of clone CLCuDβ03-PK has over 95% nucleotide sequence identity to CLCuDβ04-PK and differs from this mainly in the absence of an intact βC1 coding region due to the presence of an internal stop codon. The A-rich region of CLCuDβ03-PK (coordinates 766–98) contains 58.9% A, compared to an overall A content of 29.2%.

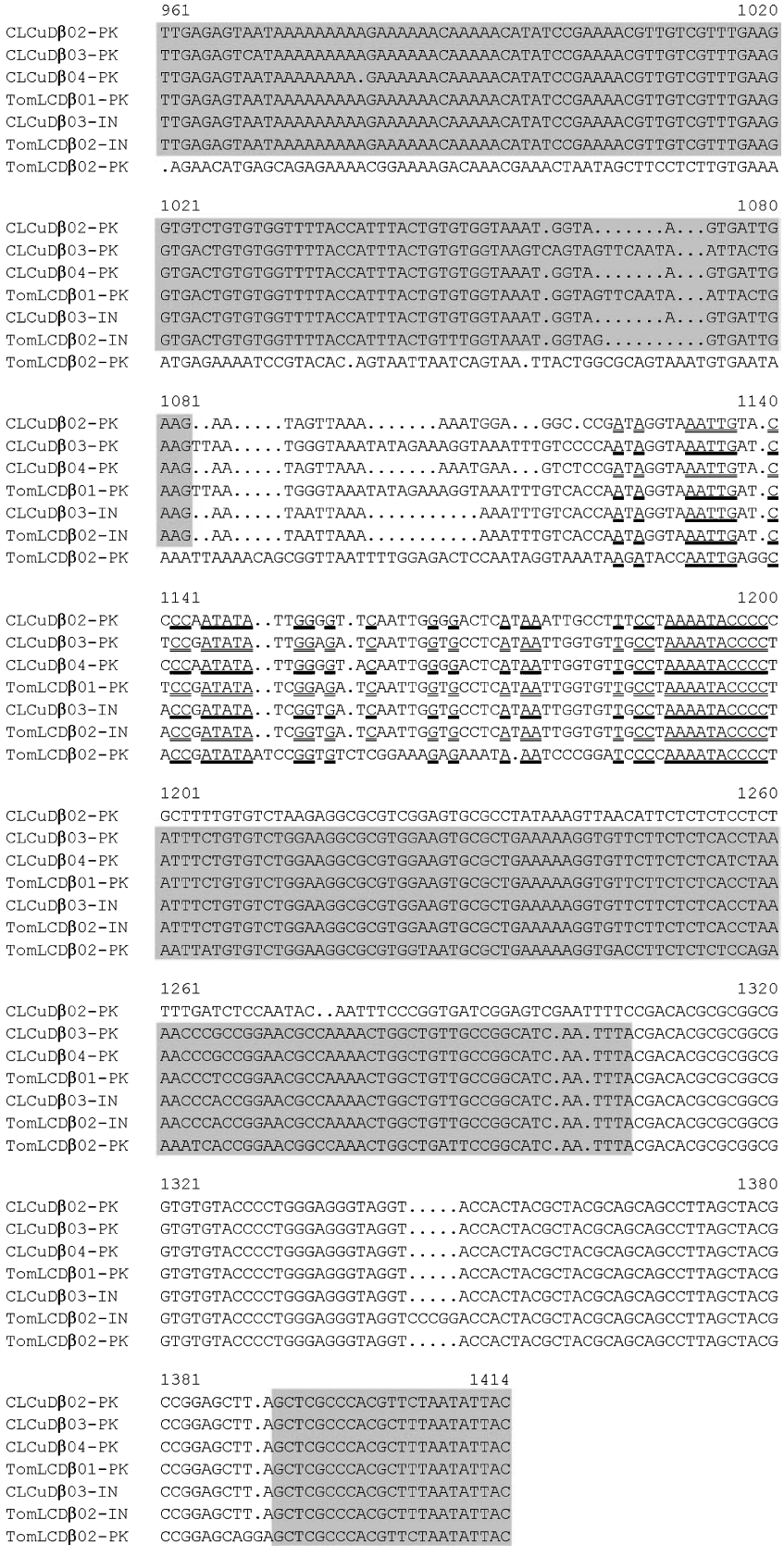
The sequences of these DNA β molecules show high levels of identity to other DNA β satellites associated with CLCuD (for example over 90% sequence identity with CLCuDβ02-PK; [1]). However, the highest levels of sequence identity are

with a DNA β associated with tomato leaf curl disease (TomLCD [4]; 95.1 and 98% sequence identity with TomLCDβ01-PK for CLCuDβ03-PK and CLCuDβ04-PK, respectively). TomLCDβ01-PK is a recombinant DNA β that consists largely of the sequences (βC1 coding region and A-rich region) originating from CLCuD DNA β but with an SCR containing sequences originating from a satellite associated with TomLCD (the comparisons here were made with ToLCDβ02-PK; [4]). Recombinant DNA β molecules, very similar to TomLCDβ01-PK, have also been isolated from both cotton (accession number AY083590) and tomato (AY438562) originating from India (we shall refer to these DNA βs as CLCuDβ03-IN and TomLCDβ02-IN, respectively).

Overall, the nucleotide sequences (excluding the SCR) of the recombinant DNA β molecules and those associated with CLCuD show over 95% identity, while that of TomLCDβ02-PK shows less than 47% identity (results not shown). These findings indicate that the DNA β isolated from CLCuD-resistant cotton is a recombinant with part of the SCR originating from a tomato DNA β satellite and the remaining sequences originating from CLCuD DNA β. We shall henceforth refer to the recombinant satellites as DNAβ^{rec}.

An alignment of the SCR regions of the DNAβ^{rec} molecules, as well as CLCuD DNA β and TomLCD DNA β, is shown in Fig. 1. The figure is of the sequences between the A-rich region (the 3′ end of the A-rich region represented by coordinates 961 to 1004 of the alignment) and the nonanucleotide sequence (coordinate 1406 of the alignment). This alignment shows that between the CLCuD DNA β-derived sequences and the probable TomLCD DNA β-originating sequences of the recombinant satellites lies a region of highly variable sequence. This contains some sequence that is common to both CLCuD and TomLCD DNA βs (double underlined) and contains the 5′ break points for the recombinations of all the DNAβ^{rec} molecules examined. The 3′ end of the recombination in each case is likely to be the (presumed) origin of virion-strand DNA replication. The origin of virion-strand replication for satellite molecules has yet to be mapped, however, the similarity between the hairpin structure of DNA β molecules and that of geminiviruses (which marks the origin of virion-sense DNA replication for these viruses; [8, 9, 18]) strongly suggests that this structure is the origin for the satellites. The geminivirus origin has, in addition, been shown to be a "hotspot" for recombination [12]. The source of the sequence variation within the SCR of the recombinant satellites is unclear. Either this indicates that each of the recombinant DNA βs has resulted from interaction with a distinct TomLCD DNA β-like satellite or alternatively that this region of the SCR is a mutation "hot-spot". Its position immediately downstream of the A-rich region may be a factor. Possibly during virion(+)-strand replication the A-rich sequence leads to a localized depletion of adenine, leading to subsequent misincorporation in this area of the genome.

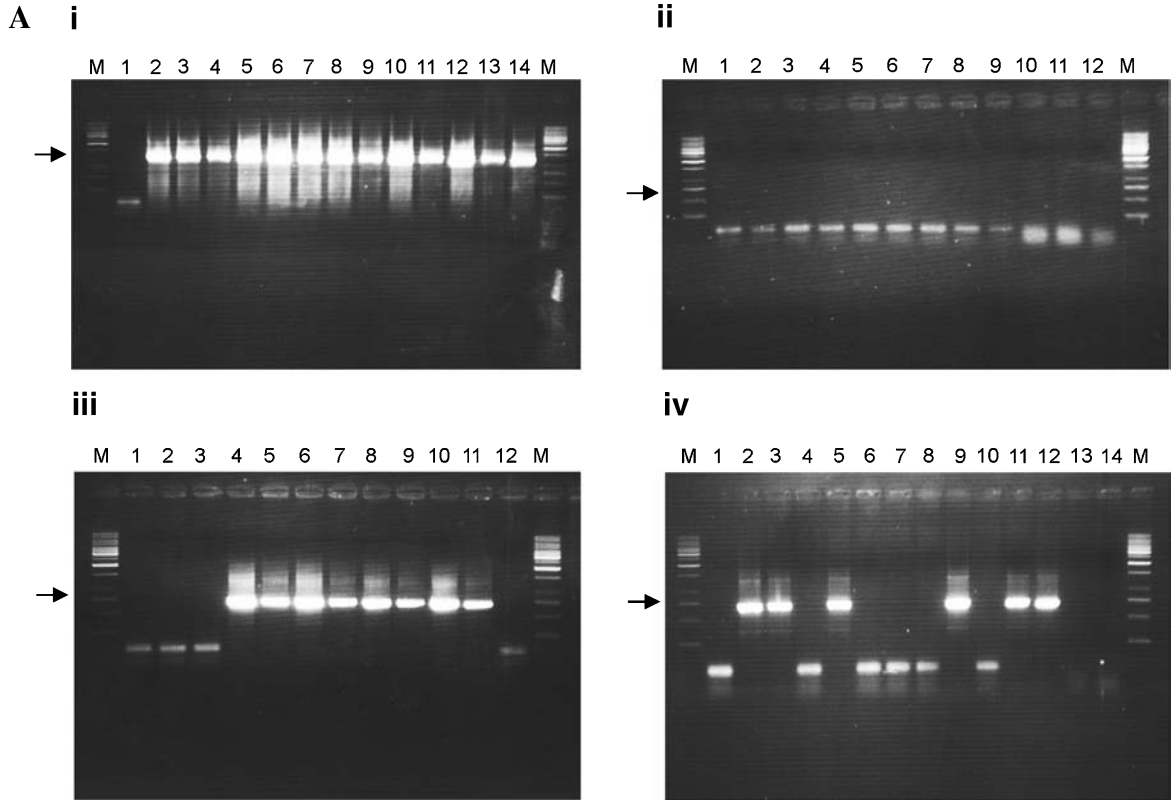
The infectivity of both CLCuDβ03-PK and TomLCuDβ01-PK to cotton and *N. benthamiana* was assessed by coinoculation with cotton leaf curl Kokhran virus (CLCuKV; clone pCLCV002 [11]). Both molecules were able to infect these hosts (14 out of 30 inoculated *N. benthamiana* and 15 of 46 inoculated *G. hirsutum* cv. S12 infected for CLCuDβ04-PK; 10 out of 20 inoculated *N. benthamiana* and

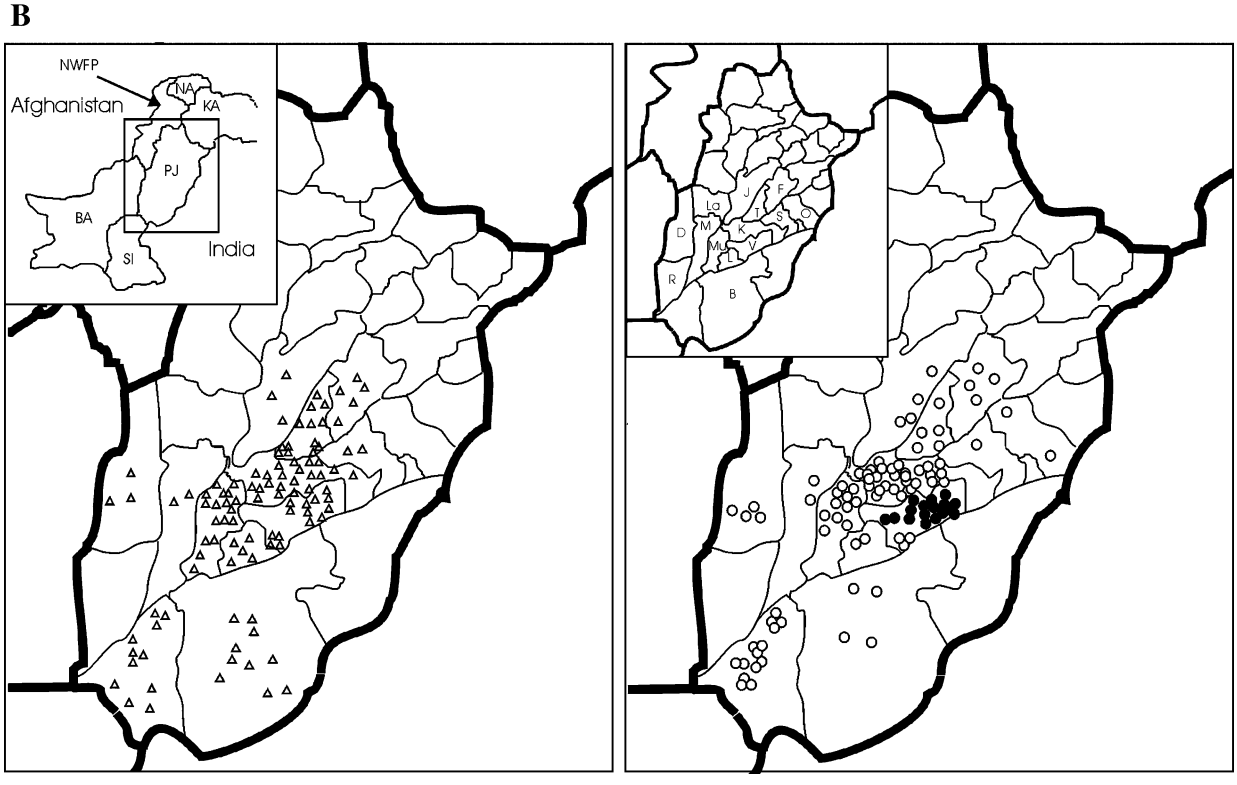

961 1020
CLCuDβ02-PK TTGAGAGTAATAAAAAAAAAGAAAAAACAAAAACATATCCGAAAACGTTGTCGTTTGAAG
CLCuDβ03-PK TTGAGAGTCATAAAAAAAAAGAAAAAACAAAAACATATCCGAAAACGTTGTCGTTTGAAG
CLCuDβ04-PK TTGAGAGTAATAAAAAAAA.GAAAAAACAAAAACATATCCGAAAACGTTGTCGTTTGAAG
TomLCDβ01-PK TTGAGAGTAATAAAAAAAAAGAAAAAACAAAAACATATCCGAAAACGTTGTCGTTTGAAG
CLCuDβ03-IN TTGAGAGTAATAAAAAAAAAGAAAAAACAAAAACATATCCGAAAACGTTGTCGTTTGAAG
TomLCDβ02-IN TTGAGAGTAATAAAAAAAAAGAAAAAACAAAAACATATCCGAAAACGTTGTCGTTTGAAG
TomLCDβ02-PK .AGAACATGAGCAGAGAAAACGGAAAAGACAAACGAAACTAATAGCTTCCTCTTGTGAAA
1021 1080
CLCuDβ02-PK GTGTCTGTGTGGTTTTACCATTTACTGTGTGGTAAAT.GGTA.......A...GTGATTG
CLCuDβ03-PK GTGACTGTGTGGTTTTACCATTTACTGTGTGGTAAGTCAGTAGTTCAATA...ATTACTG
CLCuDβ04-PK GTGACTGTGTGGTTTTACCATTTACTGTGTGGTAAAT.GGTA.......A...GTGATTG
TomLCDβ01-PK GTGACTGTGTGGTTTTACCATTTACTGTGTGGTAAAT.GGTAGTTCAATA...ATTACTG
CLCuDβ03-IN GTGACTGTGTGGTTTTACCATTTACTGTGTGGTAAAT.GGTA.......A...GTGATTG
TomLCDβ02-IN GTGACTGTGTGGTTTTACCATTTACTGTTTGGTAAAT.GGTAG.........GTGATTG
TomLCDβ02-PK ATGAGAAAATCCGTACAC.AGTAATTAATCAGTAA.TTACTGGCGCAGTAAATGTGAATA
1081 1140
CLCuDβ02-PK AAG..AA.....TAGTTAAA.......AAATGGA...GGC.CCGATAGGTAAATTGTA.C
CLCuDβ03-PK AAGTTAA.....TGGGTAAATATAGAAAGGTAAATTTGTCCCCAATAGGTAAATTGAT.C
CLCuDβ04-PK AAG..AA.....TAGTTAAA.......AAATGAA...GTCTCCGATAGGTAAATTGTA.C
TomLCDβ01-PK AAGTTAA.....TGGGTAAATATAGAAAGGTAAATTTGTCACCAATAGGTAAATTGAT.C
CLCuDβ03-IN AAG..AA.....TAATTAAA...........AAATTTGTCACCAATAGGTAAATTGAT.C
TomLCDβ02-IN AAG..AA.....TAATTAAA...........AAATTTGTCACCAATAGGTAAATTGAT.C
TomLCDβ02-PK AAATTAAAACAGCGGTTAATTTTGGAGACTCCAATAGGTAAATAAGATACCAATTGAGGC
1141 1200
CLCuDβ02-PK CCCAATATA..TTGGGGT.TCAATTGGGGACTCATAAATTGCCTTTCCTAAAATACCCCC
CLCuDβ03-PK TCCGATATA..TTGGAGA.TCAATTGGTGCCTCATAATTGGTGTTGCCTAAAATACCCCT
CLCuDβ04-PK CCCAATATA..TTGGGGT.ACAATTGGGGACTCATAATTGGTGTTGCCTAAAATACCCCT
TomLCDβ01-PK TCCGATATA..TCGGAGA.TCAATTGGTGCCTCATAATTGGTGTTGCCTAAAATACCCCT
CLCuDβ03-IN ACCGATATA..TCGGTGA.TCAATTGGTGCCTCATAATTGGTGTTGCCTAAAATACCCCT
TomLCDβ02-IN ACCGATATA..TCGGTGA.TCAATTGGTGCCTCATAATTGGTGTTGCCTAAAATACCCCT
TomLCDβ02-PK ACCGATATAATCCGGTGTCTCGGAAAGAGAAATA.AATCCCGGATCCCCAAAATACCCCT
1201 1260
CLCuDβ02-PK GCTTTTGTGTCTAAGAGGCGCGTCGGAGTGCGCCTATAAAGTTAACATTCTCTCTCCTCT
CLCuDβ03-PK ATTTCTGTGTCTGGAAGGCGCGTGGAAGTGCGCTGAAAAAGGTGTTCTTCTCTCACCTAA
CLCuDβ04-PK ATTTCTGTGTCTGGAAGGCGCGTGGAAGTGCGCTGAAAAAGGTGTTCTTCTCTCATCTAA
TomLCDβ01-PK ATTTCTGTGTCTGGAAGGCGCGTGGAAGTGCGCTGAAAAAGGTGTTCTTCTCTCACCTAA
CLCuDβ03-IN ATTTCTGTGTCTGGAAGGCGCGTGGAAGTGCGCTGAAAAAGGTGTTCTTCTCTCACCTAA
TomLCDβ02-IN ATTTCTGTGTCTGGAAGGCGCGTGGAAGTGCGCTGAAAAAGGTGTTCTTCTCTCACCTAA
TomLCDβ02-PK AATTATGTGTCTGGAAGGCGCGTGGTAATGCGCTGAAAAAGGTGACCTTCTCTCTCCAGA
1261 1320
CLCuDβ02-PK TTTGATCTCCAATAC..AATTTCCCGGTGATCGGAGTCGAATTTTCCGACACGCGCGGCG
CLCuDβ03-PK AACCCGCCGGAACGCCAAAACTGGCTGTTGCCGGCATC.AA.TTTACGACACGCGCGGCG
CLCuDβ04-PK AACCCGCCGGAACGCCAAAACTGGCTGTTGCCGGCATC.AA.TTTACGACACGCGCGGCG
TomLCDβ01-PK AACCCTCCGGAACGCCAAAACTGGCTGTTGCCGGCATC.AA.TTTACGACACGCGCGGCG
CLCuDβ03-IN AACCCACCGGAACGCCAAAACTGGCTGTTGCCGGCATC.AA.TTTACGACACGCGCGGCG
TomLCDβ02-IN AACCCACCGGAACGCCAAAACTGGCTGTTGCCGGCATC.AA.TTTACGACACGCGCGGCG
TomLCDβ02-PK AAATCACCGGAACGGCCAAACTGGCTGATTCCGGCATC.AA.TTTACGACACGCGCGGCG
1321 1380
CLCuDβ02-PK GTGTGTACCCCTGGGAGGGTAGGT.....ACCACTACGCTACGCAGCAGCCTTAGCTACG
CLCuDβ03-PK GTGTGTACCCCTGGGAGGGTAGGT.....ACCACTACGCTACGCAGCAGCCTTAGCTACG
CLCuDβ04-PK GTGTGTACCCCTGGGAGGGTAGGT.....ACCACTACGCTACGCAGCAGCCTTAGCTACG
TomLCDβ01-PK GTGTGTACCCCTGGGAGGGTAGGT.....ACCACTACGCTACGCAGCAGCCTTAGCTACG
CLCuDβ03-IN GTGTGTACCCCTGGGAGGGTAGGT.....ACCACTACGCTACGCAGCAGCCTTAGCTACG
TomLCDβ02-IN GTGTGTACCCCTGGGAGGGTAGGTCCCGGACCACTACGCTACGCAGCAGCCTTAGCTACG
TomLCDβ02-PK GTGTGTACCCCTGGGAGGGTAGGT.....ACCACTACGCTACGCAGCAGCCTTAGCTACG
1381 1414
CLCuDβ02-PK CCGGAGCTT.AGCTCGCCCACGTTCTAATATTAC
CLCuDβ03-PK CCGGAGCTT.AGCTCGCCCACGCTTTAATATTAC
CLCuDβ04-PK CCGGAGCTT.AGCTCGCCCACGCTTTAATATTAC
TomLCDβ01-PK CCGGAGCTT.AGCTCGCCCACGCTTTAATATTAC
CLCuDβ03-IN CCGGAGCTT.AGCTCGCCCACGCTTTAATATTAC
TomLCDβ02-IN CCGGAGCTT.AGCTCGCCCACGCTTTAATATTAC
TomLCDβ02-PK CCGGAGCAGGAGCTCGCCCACGTTCTAATATTAC

13 of 48 inoculated *G. hirsutum* cv. S12 infected for TomLCDβ01-PK), in the presence of the CLCuKV helper, to induce symptoms typical of CLCuD (in each case the results were of three independent infectivity experiments). In *N. benthamiana* these symptoms consisted of downward leaf curling, crumpling and, in the later stages, yellowing of the plants. For cotton, the symptoms were typically downward leaf curling, vein swelling, vein darkening and the formation of leaf-like enations on the veins on the undersides of leaves, as has previously been reported for the cloned components of the CLCuD complex [1, 11]. However, both recombinant DNA β were marginally more infectious than CLCuDβ02-PK when coinoculated with CLCuKV, and the symptoms appeared one to two days earlier, typically in 14 to 16 days compared to 18 to 20 days for clone CLCuDβ02-PK in the presence of CLCuKV (7 out of 30 inoculated *N. benthamiana* and 5 of 31 inoculated *G. hirsutum* cv. S12 infected for CLCuDβ04-PK). However, the symptom severity for infections involving the recombinant satellites were similar to those involving CLCuD DNA β, and the levels of viral and satellite DNA in infected plants, as judged by Southern blot hybridization, showed no significant differences (results not shown).

In order to study the geographical extent of the recombinant satellite associated with CLCuD in cotton, specific primers were designed (virion-sense primer 5′GTGACTCGAGTCTTCGTACGTGTACTAGACG 3′; complementary-sense primer 5′GTCGCCATGGGAGATCAATTTACCTATTGGG3′). The primers were shown not to amplify a product from healthy cotton plants or cotton plants experimentally infected with CLCuKV and CLCuD DNA β clone CLCuDβ02-Pak. Nucleic acid extracts of 112 cotton samples, collected between 1995 and 1999 from cotton-growing areas across Pakistan, were tested for the presence of the recombinant β (Fig. 2A, Table 1). Only samples were included from which a full-length product (of approx. 1400 nucleotides) could be amplified in PCR reactions with universal DNA β primers [2]. None of these samples tested positive for the presence of recombinant β. In contrast, a similar analysis of 106 samples collected during years 2001 to 2003 from the area where the resistance-breaking strain is prevalent showed that the recombinant β was confined to these districts only (Fig. 2B). This is strong evidence suggesting that mobilisation of the recombinant

Fig. 1. Alignment of the satellite conserved regions of the DNA β molecules isolated from cotton leaf curl-resistant cotton, a typical CLCuD DNA β (CLCuDβ02-PK), a typical TomLCD DNA β (TomLCDβ02-PK) and recombinant DNA β molecules previously isolated from tomato (TomLCDβ01-PK and TomLCDβ02-IN) and cotton (CLCuDβ03-IN). Sequences homologous to CLCuD DNA β are highlighted in grey at the 5′ end (alignment coordinates 961–1083). After this (1084–1200) is a region of high sequence variability in which the nucleotide sequences similar to CLCuD DNA β are double underlined. Sequences from 1201 to 1305 are homologous to TomLCD DNA β (highlighted in grey). Sequences from 1206 onwards are conserved between all the DNA β molecules shown and include the conserved hairpin structure (only the left leg of which is shown) and nonanucleotide sequence (highlighted in grey; 1392–1414)


A
i
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 M
ii
M 1 2 3 4 5 6 7 8 9 10 11 12 M
iii
M 1 2 3 4 5 6 7 8 9 10 11 12 M
iv
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 M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DNA β into cotton, at least in the area examined, is recent and that until 2003 this was confined to a limited area in Vehari district. This has likely occurred due to the polyphagous nature of the vector of all begomoviruses, the whitefly *B. tabaci*. The recombination event(s) leading to CLCuD DNA β^{rec} likely occurred in a host other than cotton, such as tomato, since TomLCD DNA β does not support virus infection of cotton (unpublished results). The CLCuD complex, however, is able to symptomatically infect tomato (unpublished results), while both CLCuD DNA β^{rec} and TomLCD DNA β^{rec} can interact with at least one of the CLCuD-associated begomoviruses (CLCuKV) to symptomatically infect cotton.

Identification of similar recombinant DNA β molecules in both cotton and tomato in India indicates that interactions between the TomLCD and CLCuD begomovirus complexes are widespread and that the SCR is a "hotspot" for recombination. Whether the recombinant DNA β is associated with resistance breaking in India is not known, although resistance breaking has now also been reported there. The precise mechanism of resistance breaking, as well as the role played by CLCuD DNA β^{rec} in this, remains to be determined. All attempts to inoculate CLCuDβ04-PK to resistant cotton varieties in the presence of CLCuKV,

Fig. 2. PCR-mediated detection of DNA β using universal DNA β primers (panel **Ai**) and the recombinant DNA β (using specific primers; **Aii**, **iii** and **iv**). Samples run on the gels resulted from PCR reactions containing nucleic acids extracted from leaf samples collected between 1995 and 1999 (**i** and **ii**) and between 2000 and 2003 (**iii** and **iv**). For panel **Ai** the sample in *1* and *2* resulted from PCR reactions containing nucleic acids extracted from a healthy cotton plant and a cotton plant infected with CLCuKV and CLCuD DNA β (confirmed by cloning and sequence analysis). Samples in *3* to *14* resulted from PCR reactions containing DNA extracted from samples 23a, 58a, 28a, 33a, 60a, 39a, 41a, 66a, 67a, 9a, 17a and 103a (Table 1), respectively. For panel **Aii**, DNA extracted from samples 23a, 58a, 28a, 33a, 60a, 39a, 41a, 66a, 67a, 9a, 17a and 103a, respectively, was used as template in PCR reactions. In panel **Aiii**, samples were extracted from a healthy cotton plant, a cotton plant infected with CLCuKV and CLCuD DNA β, a cotton plant infected with CLCuD DNA β^{rec} and an unidentified begomovirus (confirmed by sequence analysis), 25b, 61b, 26b, 27b, 36b, 82b, 24b, 58b for *1* to *14*, respectively, but excluding *4*. The template used for the PCR in *4* was diluted plasmid clone CLCuVβ02-PK (CLCuD DNA β). For panel **Aiv** the samples were extracted from healthy cotton, a cotton plant infected with CLCuD DNA β^{rec}, 57b, 2b, 105b, 52b, 85b, 104b, 71b, 64b, 8b, 56b, 66b and 74b, respectively. In each case, the lanes marked *M* contained a 1-kb DNA ladder (Fermentas). The size of the expected product is shown by an arrow for each gel. **B** shows a map of Punjab province with districts (inset right). The origins of CLCuD-infected cotton samples collected from 1995 to 1999 (left) and between 2000 and 2003 (right) are shown. For the samples collected after 1999 (right), those testing positive for the presence of CLCuD β^{rec} are shown as black circles, whereas those testing negative are shown as white circles. Provinces (inset left) are abbreviated as Bahawalpur (*BA*), Kashmir (*KA*), Northern Areas (*NA*), Punjab (*PJ*), North West Frontier Province (*NWFP*) and Sindh (*SI*). Districts (inset left) are abbreviated as Faisalabad (*F*), Jhang (*J*), Layyah (*La*), Lodhra (*L*), Toba Tek Singh (*T*), Khanewal (*K*), Okara (*O*), Vehari (*V*), Multan (*Mu*), Muzafargarh (*M*), Rahim Yaar Khan (*R*), Bahawalpur (*B*), Sahiwal (*S*) and Dear Ghazi Khan (*D*)

papaya leaf curl virus or cotton leaf curlMultan virus (the only clones shown to be infectious to cotton [1, 11]) were unsuccessful (results not shown), possibly suggesting that CLCuD DNA β^{rec} is not responsible for resistance breaking. However, our biolistic inoculation procedure for cotton is not sufficiently robust to

Table 1. Detection of DNA β in and origins of samples collected between 1995 and 2003

Sample no.	Origin town (district[a])	Variety[b]	Year of collection	β*	β^{rec}*	Sample no.	Origin town (district[a])	Variety[b]	Year of collection	β*	β^{rec}*
9a	Ahmed Pur Sharqia (R)	ND[S]	1998	+	−	102a	Lodhraan (T)	ND[S]	1995	+	−
20a	Ahmed Pur Sharqia (R)	ND[S]	1996	+	−	108a	Lodhraan (T)	ND[S]	1995	+	−
63a	Ahmed Pur Sharqia (R)	ND[S]	1995	+	−	53a	Mailse (V)	ND[S]	1998	+	−
84a	Ahmed Pur Sharqia (R)	ND[S]	1999	+	−	72a	Mailse (V)	ND[S]	1995	+	−
3a	(B)	ND[S]	1996	+	−	91a	Mailse (V)	ND[S]	1996	+	−
21a	(B)	ND[S]	1998	+	−	92a	Mailse (V)	ND[S]	1999	+	−
30a	(B)	ND[S]	1997	+	−	17a	Mianchunu (K)	ND[S]	1997	+	−
38a	(B)	ND[S]	1996	+	−	35a	Mianchunu (K)	ND[S]	1997	+	−
64a	(B)	ND[S]	1997	+	−	2a	(M)	ND[S]	1995	+	−
66a	(B)	ND[S]	1998	+	−	7a	(M)	ND[S]	1998	+	−
107a	(B)	ND[S]	1995	+	−	11a	(M)	ND[S]	1996	+	−
29a	(B)	ND[S]	1995	+	−	13a	(M)	ND[S]	1997	+	−
50a	(B)	ND[S]	1998	+	−	23a	(M)	ND[S]	1996	+	−
82a	(B)	ND[S]	1998	+	−	25a	(M)	ND[S]	1996	+	−
88a	(B)	ND[S]	1995	+	−	46a	(M)	ND[S]	1995	+	−
43a	Burewala (V)	ND[S]	1996	+	−	56a	(M)	ND[S]	1998	+	−
111a	Burewala (V)	ND[S]	1996	+	−	70a	(M)	ND[S]	1995	+	−
90a	Burewla (V)	ND[S]	1997	+	−	86a	(M)	ND[S]	1998	+	−
24a	(K)	ND[S]	1997	+	−	87a	(M)	ND[S]	1995	+	−
10a	(K)	ND[S]	1996	+	−	110a	(M)	ND[S]	1996	+	−
75a	(K)	ND[S]	1995	+	−	112a	(M)	ND[S]	1999	+	−
18a	Cheechawatni (S)	ND[S]	1996	+	−	81a	(Mz)	ND[S]	1999	+	−
28a	Cheechawatni (S)	ND[S]	1998	+	−	47a	(Mz)	ND[S]	1996	+	−
52a	Cheechawatni (S)	ND[S]	1997	+	−	54a	Peerowal (K)	ND[S]	1997	+	−
73a	Cheechawatni (S)	ND[S]	1998	+	−	79a	Peerowal (K)	ND[S]	1995	+	−
94a	Cheechawatni (S)	ND[S]	1996	+	−	80a	Peerowal (K)	ND[S]	1998	+	−
15a	(D)	ND[S]	1995	+	−	105a	Peerowal (K)	ND[S]	1996	+	−
27a	(D)	ND[S]	1997	+	−	14a	(R)	ND[S]	1997	+	−
65a	(D)	ND[S]	1998	+	−	31a	(R)	ND[S]	1996	+	−
62a	(F)	ND[S]	1995	+	−	33a	(R)	ND[S]	1999	+	−
99a	(F)	ND[S]	1997	+	−	44a	(R)	ND[S]	1998	+	−
100a	(F)	ND[S]	1999	+	−	12a	Rajana (T)	ND[S]	1995	+	−
37a	(J)	ND[S]	1999	+	−	58a	Rajana (T)	ND[S]	1997	+	−
61a	(J)	ND[S]	1998	+	−	77a	Rajana (T)	ND[S]	1998	+	−
96a	(J)	ND[S]	1997	+	−	93a	Rajana (T)	ND[S]	1995	+	−
36a	Kabirwala (M)	ND[S]	1997	+	−	57a	Rukanpur (R)	ND[S]	1997	+	−
103a	Kabirwala (M)	ND[S]	1997	+	−	59a	(S)	ND[S]	1995	+	−
42a	Kamalia (T)	ND[S]	1999	+	−	104a	(S)	ND[S]	1996	+	−
69a	Kamalia (T)	ND[S]	1997	+	−	49a	Shujaabad (M)	ND[S]	1999	+	−
95a	Kamalia (T)	ND[S]	1996	+	−	71a	Shujaabad (M)	ND[S]	1999	+	−
6a	Katcha Khu (K)	ND[S]	1996	+	−	19a	Summandri (F)	ND[S]	1996	+	−
55a	Katcha khu (K)	ND[S]	1997	+	−	22a	Talamba (K)	ND[S]	1998	+	−
83a	Katcha Khu (K)	ND[S]	1995	+	−	67a	Talamba (K)	ND[S]	1996	+	−
26a	(K)	ND[S]	1995	+	−	106a	Talamba (K)	ND[S]	1997	+	−
32a	(K)	ND[S]	1998	+	−	98a	Theekriwala (F)	ND[S]	1999	+	−
39a	(K)	ND[S]	1996	+	−	5a	(T)	ND[S]	1997	+	−
40a	(K)	ND[S]	1999	+	−	8a	(T)	ND[S]	1998	+	−
76a	(K)	ND[S]	1997	+	−	60a	(T)	ND[S]	1995	+	−
101a	(K)	ND[S]	1998	+	−	97a	(T)	ND[S]	1998	+	−
1a	Khanpur (R)	ND[S]	1995	+	−	4a	(V)	ND[S]	1995	+	−
45a	Khanpur (R)	ND[S]	1996	+		16a	(V)	ND[S]	1998	+	−
78a	Khanpur (R)	ND[S]	1998	+	−	41a	(V)	ND[S]	1996	+	−
51a	Khokraan (M)	ND[S]	1997	+	−	48a	(V)	ND[S]	1997	+	−
34a	Lodhraan (T)	ND[S]	1997	+	−	74a	(V)	ND[S]	1995	+	−
68a	Lodhraan (T)	ND[S]	1997	+	−	89a	(V)	ND[S]	1998	+	−
85a	Lodhraan (T)	ND[S]	1998	+	−	109a	(V)	ND[S]	1998	+	−

(*continued*)

Table 1 (*continued*)

Sample no.	Origin town (district[a])	Variety[b]	Year of collection	β*	β^{rec}*	Sample no.	Origin town (district[a])	Variety[b]	Year of collection	β*	β^{rec}*
17b	Ahmed Pur Sharqia (R)	BH-36[S]	2001	+	–	66b	Lodhraan (T)	ND	2003	+	–
68b	Ahmed Pur Sharqia (R)	LRA-5166[R]	2002	+	–	93b	Lodhraan (T)	ND	2003	+	–
72b	Ahmed Pur Sharqia (R)	N D	2003	+	–	26b	Luddan (V)	CP 15/2[R]	2002	+	+
42b	Ahmed Pur Sharqia (R)	NIAB-78[S]	2003	+	–	57b	Luddan (V)	NIAB-111[S]	2002	+	+
5b	Amangarh (R)	CIM 109[S]	2002	+	–	58b	Mailsee (V)	Karishma[S]	2003	+	–
14b	(B)	BH-36[S]	2002	+	–	84b	Mailsee (V)	LRA-5166[R]	2003	+	–
52b	(B)	CP 15/2[R]	2003	+	–	43b	Mailsee (V)	NIAB-98[S]	2003	+	–
81b	(B)	BH-36[S]	2001	+	–	6b	Mianchanu (K)	Mix[S]	2001	+	–
45b	Basti Rehmatullah (V)	ND	2003	+	–	75b	Mianchunu (K)	Karishma[S]	2001	+	–
25b	Burewala (V)	N D	2001	+	+	31b	Mianchunu (K)	Karishma[S]	2001	+	–
36b	Burewala (V)	LRA-5166[R]	2002	+	+	15b	(M)	ND		+	–
27b	Burewala (V)	LRA-5166[R]	2002	+	+	20b	(M)	CIM-446[S]	2002	+	–
8b	Burewala (V)	CP 15/2[R]	2002	+	+	39b	(M)	CIM-473[S]	2001	+	–
82b	Burewala (V)	CIM-446[S]	2002	+	+	55b	(M)	N.D	2002	+	–
105b	Burewala (V)	CIM-449[S]	2003	+	+	67b	(M)	N.D	2003	+	–
41b	B. Mugarana (V)	CP 15/2[R]	2001	+	+	95b	(M)	CIM-446[S]	2003	+	–
76b	Cheechawatni (Sa)	N.D	2003	+	–	99b	(M)	CIM473[S]	2002	+	–
38b	(D)	BH-95[S]	2003	+	–	64b	(Mz)	NIAB 111[S]	2001	+	–
50b	(D)	BH-36[S]	2002	+	–	19b	(Mz)	CIM-446[S]	2002	+	–
79b	(D)	ND	2003	+	–	77b	(O)	ND	2001	+	–
92b	(D)	ND	2001	+	–	37b	(K)	ND	2002	+	–
18b	Daimwala (V)	BH-95[S]	2001	+	–	47b	(K)	NIAB 111[S]	2001	+	–
22b	(F)	NIAB-111[S]	2003	+	–	91b	(K)	Mix[S]	2002	+	–
53b	(F)	Karishma[S]	2001	+	–	60b	Qatalpur (J)	Karishma[S]	2002	+	–
54b	(F)	NIAB 98[S]	2002	+	–	12b	(R)	BH-36[S]	2002	+	–
106b	(F)	ND	2003	+	–	40b	(R)	ND	2001	+	–
9b	Jahania (L)	CIM-497[S]	2002	+	–	44b	(R)	BH-36[S]	2003	+	–
48b	Jahania (L)	CIM497[S]	2003	+	–	49b	(R)	ND	2002	+	–
73b	Jahania (L)	ND	2001	+	–	63b	(R)	ND	2001	+	–
102b	Jahania (L)	BH-36[S]	2002	+	–	80b	(R)	ND	2003	+	–
59b	(J)	NIAB-111[S]	2001	+	–	94b	(R)	BH-95[S]	2001	+	–
97b	(J)	ND	2001	+	–	104b	(R)	CIM-446[S]	2002	+	–
23b	(J)	Karishma[S]	2001	+	–	32b	(R)	ND	2003	+	–
34b	(J)	ND	2003	+	–	85b	Rajana (T)	ND	2001	+	–
2b	Kabirwala (M)	Karishma[S]	2001	+	–	3b	Rukanpur (R)	Karishma[S]	2003	+	–
11b	Kabirwala (M)	Karishma[S]	2003	+	–	29b	Rukanpur (R)	ND	2003	+	–
70b	Kabirwala (M)	Karishma[S]	2003	+	–	87b	Rukanpur (R)	ND	2002	+	–
100b	Kabirwala (M)	Karishma[S]	2003	+	–	46b	(Sa)	NIAB-111[S]	2002	+	–
86b	Kamalia (T)	NIAB-78[S]	2002	+	–	78b	(Sa)	NIAB-111[S]	2002	+	–
21b	Katcha Khu (K)	LRA-5166[R]	2002	+	–	4b	Saidpur J.D.W. (B)	BH-36[S]	2003	+	–
90b	Katcha Khu (K)	NIAB 111[S]	2003	+	–	65b	Shujaabad (M)	ND	2001	+	–
98b	Katcha Khu (K)	ND	2001	+	–	69b	Sialwala (M)	ND	2002	+	–
1b	(K)	Karishma[S]	2001	+	–	88b	Talamba (K)	NIAB-98[S]	2001	+	–
10b	(K)	Karishma[S]	2001	+	–	101b	Talamba (K)	ND	2001	+	–
16b	(K)	Karishma[S]	2003	+	–	35b	(T)	NIAB 111[S]	2001	+	–
62b	(K)	Karishma[S]	2001	+	–	96b	(T)	NIAB 111[S]	2003	+	–
89b	(K)	Karishma[S]	2003	+	–	7b	(V)	LRA-5166[R]	2001	+	+
103b	(K)	Karishma[S]	2002	+	–	13b	(V)	LRA-5166[R]	2003	+	+
28b	(K)	Karishma[S]	2003	+	–	24b	(V)	CIM-446[S]	2002	+	+
30b	(K)	Karishma[S]	2003	+	–	56b	(V)	CP 15/2[R]	2002	+	+
33b	(K)	Karishma[S]	2002	+	–	61b	(V)	CP 15/2[R]	2003	+	+
74b	(L)	NIAB 111[S]	2003	+	–	71b	(V)	LRA-5166[R]	2003	+	+
51b	Lodhraan (T)	BH-36[S]	2003	+	–	83b	(V)	CP 15/2[R]	2002	+	+

[a]The abbreviations for districts are as given for Fig. 2

[b]Cotton varieties are denoted as either susceptible (S) or resistant (R)

*Results for the PCR-mediated detection of either DNA β (β) or DNA β^{rec} (β^{rec})

ND not determined

be able to draw any firm conclusions from our inability to infect resistant varieties with these clones. Additionally, during 2004 we identified CLCuD in resistant cotton varieties that were not associated with the recombinant DNA β molecule. Whether this is due to additional strains capable of resistance breaking evolving or is due to further changes in the established resistance-breaking strain (possibly by additional recombination events) remains to be determined. These findings do, however, show that the situation with CLCuD is in flux, with further changes to the complex occurring in the field.

We have previously shown that DNA β satellites found in malvaceous hosts are distinct from those infecting other plant families [4]. Our findings indicate that recombination between distinct DNA β satellites is a further mechanism of generating diversity that may assist geminiviruses in adapting/evolving. Our efforts now centre on isolating clones of the begomovirus(es) associated with the resistance-breaking strain of CLCuD from the Vehari area (associated with CLCuD DNA β^{rec}) to establish whether these are experimentally infectious to resistant cotton varieties. The aim is, ultimately, to define the precise molecular basis for resistance breaking.

References

1. Briddon RW, Mansoor S, Bedford ID, Pinner MS, Saunders K, Stanley J, Zafar Y, Malik KA, Markham PG (2001) Identification of DNA components required for induction of cotton leaf curl disease. Virology 285: 234–243
2. Briddon RW, Bull SE, Mansoor S, Amin I, Markham PG (2002) Universal primers for the PCR-mediated amplification of DNAβ; a molecule associated with some monopartite begomoviruses. Mol Biotechnol 20: 315–318
3. Briddon RW (2003) Cotton leaf curl disease, a multicomponent begomovirus complex. Mol Plant Pathol 4: 427–434
4. Briddon RW, Bull SE, Amin I, Idris AM, Mansoor S, Bedford ID, Dhawan P, Rishi N, Siwatch SS, Abdel-Salam AM, Brown JK, Zafar Y, Markham PG (2003) Diversity of DNA β: a satellite molecule associated with some monopartite begomoviruses. Virology 312: 106–121
5. Briddon RW, Stanley J (2006) Sub-viral agents associated with plant-infecting single-stranded DNA viruses. Virology 344: 198–210
6. Cui X, Tao X, Xie Y, Fauquet CM, Zhou X (2004) A DNA β associated with *Tomato yellow leaf curl China virus* is required for symptom induction. J Virol 78: 13966–13974
7. Cui X, Li G, Wang D, Hu D, Zhou X (2005) A begomovirus DNA β-encoded protein binds DNA, functions as a suppressor of RNA silencing, and targets the cell nucleus. J Virol 79: 10764–10775
8. Heyraud F, Matzeit V, Kammann M, Schafer S, Schell J, Gronenborn B (1993) Identification of the initiation sequence for viral-strand DNA synthesis of wheat dwarf virus. EMBO J 12: 4445–4452
9. Heyraud F, Matzeit V, Schaefer S, Schell J, Gronenborn B (1993) The conserved nonanucleotide motif of the geminivirus stem-loop sequence promotes replicational release of virus molecules from redundant copies. Biochimie 75: 605–615
10. Mansoor S, Amin I, Iram S, Hussain M, Zafar Y, Malik KA, Briddon RW (2003) The breakdown of resistance in cotton to cotton leaf curl disease in Pakistan. New Disease Reports vol 7, [http://www.bspp.org.uk/ndr/]

11. Mansoor S, Briddon RW, Bull SE, Bedford ID, Bashir A, Hussain M, Saeed, Zafar MY, Malik KA, Fauquet C, Markham PG (2003) Cotton leaf curl disease is associated with multiple monopartite begomoviruses supported by single DNA β. Arch Virol 148: 1969–1986
12. Padidam M, Sawyer S, Fauquet CM (1999) Possible emergence of new geminiviruses by frequent recombination. Virology 265: 218–225
13. Rahman H, Khan WS, Khan M, Shah MKN (2001) Stability of cotton cultivars under leaf curl virus epidemic in Pakistan. Field Crops Res 69: 251–257
14. Rahman M, Hussain D, Malik TA, Zafar Y (2005) Genetics of resistance against cotton leaf curl disease in *Gossypium hirsutum*. Plant Pathol 54: 764–772
15. Saeed M, Behjatnia SAA, Mansoor S, Zafar Y, Hasnain S, Rezaian MA (2004) A single complementary-sense transcript of a geminiviral DNA β satellite is determinant of pathogenicity. Mol Plant Microbe Interact 18: 7–14
16. Saunders K, Bedford ID, Briddon RW, Markham PG, Wong SM, Stanley J (2000) A novel virus complex causes *Ageratum* yellow vein disease. In: Proc Natl Acad Sci USA 97: 6890–6895
17. Saunders K, Norman A, Gucciardo S, Stanley J (2004) The DNA β satellite component associated with ageratum yellow vein disease encodes an essential pathogenicity protein (βC1). Virology 324: 37–47
18. Stanley J (1995) Analysis of African cassava mosaic virus recombinants suggests strand nicking occurs within the conserved nonanucleotide motif during the initiation of rolling circle DNA replication. Virology 206: 707–712
19. Stanley J, Bisaro DM, Briddon RW, Brown JK, Fauquet CM, Harrison BD, Rybicki EP, Stenger DC (2005) Geminiviridae. In: Fauquet CM, Mayo MA, Maniloff J, Desselberger U, Ball LA (eds) Virus Taxonomy, VIIIth Report of the ICTV. Elsevier/Academic Press, London, pp 301–326

Author's address: Imran Amin, National Institute for Biotechnology and Genetic Engineering (NIBGE), P.O. Box 577, Jhang Road, Faisalabad, Pakistan; e-mail: imran@nibge.org