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Tomato leaf curl geminivirus from India has a bipartite genome and coat protein is not essential for infectivity

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Genomes of two isolates of tomato leaf curl geminivirus from India (ToLCV-India) have been sequenced. ToLCV-India contains A and B components, both of which are required for systemic movement and symptom development. The two isolates have 94% sequence identity but one isolate gave mild symptoms in *Nicotiana benthamiana* and tomato. The genome organization of ToLCV-India is similar to other whitefly-transmitted geminiviruses (WTGs) with bipartite genomes. However, it contains an additional ORF, AV3, that has not been reported for other WTGs. Its coat protein (CP) sequence is highly homologous to that of Indian cassava mosaic virus (90%). Two mutations that truncated the CP after amino acids 65 or 172 did not affect systemic movement and symptom development in either *N. benthamiana* or tomato. However, the symptoms caused by mutant viruses were different from those in plants infected with unmodified viruses, and plants infected with the mutants had markedly reduced amounts of single-stranded viral DNA. Comparison of sequences and other biological features of ToLCV-India with other geminiviruses showed that ToLCV-India is a distinct virus and is related to the WTGs from the Old World. It is similar to African cassava mosaic virus in its requirement for B component and dispensability of coat protein for symptom development, unlike other geminiviruses that infect tomato in the Old World. It is proposed that ToLCV-India evolved more recently as compared to other geminiviruses that infect tomato in the Old World.

Introduction

The geminiviruses are plant viruses that have circular, single-stranded DNA genomes, packaged within geminate particles. The genome organization and biological properties of geminiviruses show that they may be divided into three subgroups (Davies & Stanley, 1989; Francki *et al.*, 1991; Lazarowitz, 1992). The geminiviruses that have monopartite genomes and are transmitted by leafhoppers to monocotyledonous plants are placed in subgroup I, of which maize streak virus (MSV) is the type species. Subgroup I also includes tobacco yellow dwarf virus (TYDV), which infects dicotyledonous plants. Subgroup II comprises viruses with monopartite genomes that are transmitted by leafhoppers to dicotyledonous plants, with beet curly top virus (BCTV) as the type species. The third subgroup includes viruses that are transmitted by whiteflies to dicotyledonous plants. These viruses have bipartite genomes (except some isolates of tomato yellow leaf curl virus) and bean golden mosaic virus (BGMV) is considered as the type species.

Several geminivirus isolates infecting tomato in the Old World have been characterized. Unlike other whitefly-transmitted geminiviruses (WTGs), tomato yellow leaf curl virus (TYLCV)-Israel (Navot *et al.*, 1991), TYLCV-Sardinia (Kheyr-Pour *et al.*, 1991) and tomato leaf curl virus (ToLCV)-Australia isolates (Dry *et al.*, 1993) do not have a B component. In contrast, a TYLCV-Thailand isolate does have a B component (Rochester *et al.*, 1990). However, in the latter case, the A component alone induces strong disease symptoms, while the B component enhances symptom severity. These tomato viruses also differ in requirement for coat protein (CP); while CP is essential for spread and symptom development in ToLCV-Australia (Rigden *et al.*, 1993), mutations that eliminate CP production delay, but do not eliminate, symptom development by TYLCV-Thailand (Rochester *et al.*, 1994). Sequence comparisons of viruses that infect tomato in the Old World show that they diverged much more than expected for strains of the same virus, and some of the isolates are more similar to

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geminiviruses that infect other plants than to isolates that infect tomato (Dry *et al.*, 1993; Hong *et al.*, 1993*b*; Kheyr-Pour *et al.*, 1991). Based on these observations it is proposed that the different geminiviruses that infect tomato evolved in different geographical regions. It is also interesting that some geminiviruses infecting tomato have absolute requirements for B component (TGMV and tomato mottle virus), while for others the B component is not present (TYLCV-Israel, TYLCV-Sardinia and ToLCV-Australia) or not required (TYLCV-Thailand). In this context it is of interest to examine the relationship of tomato leaf curl geminivirus from India (ToLCV-India) with other geminiviruses. ToLCV-India is a serious disease on tomato throughout the Indian subcontinent, limiting tomato production to seasons and locations when populations of whitefly are low. Molecular characterization of this virus is a prerequisite for selection of control strategies based on the expression of viral genes in tomato.

Methods

Cloning of the ToLCV genome. Tomato (*Lycopersicon esculentum*) plants showing symptoms of leaf curl were collected from a small experimental field in New Delhi, India in 1992 and total DNA was isolated essentially as described in Coutts *et al.* (1988). Electrophoresis of total DNA from infected and healthy plants in gels containing 1% agarose revealed that infected plants had DNA bands at 1·6 and 1·8 kb that were absent from healthy plants. S1 nuclease treatment of DNA before electrophoresis in agarose gels and Southern blot hybridization using ACMV A DNA (Stanley & Gay, 1983) as the probe indicated that the band at 1·8 kb is a double-stranded (ds) replicative form of geminivirus DNA. The dsDNA was purified from a preparative gel and digested with several restriction enzymes, and putative full length linear DNA was cloned at the *Eco*RV site in pBluescript SK(−) (Stratagene), at the *Hin*dIII site in pGEM-7zf(+) (Promega) and at the *Pst*I site in pTZ18R (Pharmacia).

Infectivity of cloned DNAs. A particle acceleration method was used to inoculate plants since ToLCV-India is not mechanically transmissible. Infectivity was confirmed by constructing partial tandem dimers (as described by Hayes *et al.*, 1988) and shooting partial dimeric DNA onto *Nicotiana benthamiana* or tomato seedlings using a Bio-Rad helium driven particle accelerator. DNA plus gold particles (0·5 μg DNA plus 0·8 mg gold particles per shot) were prepared as described by Klein *et al.* (1988) and accelerated at 1300 p.s.i. onto leaves of 2-week-old seedlings germinated on MS basal salts medium (Murashige & Skoog, 1962) in Magenta boxes (one per box). The plants were maintained at 16/8 h light/dark periods for 3–4 weeks until they were scored for symptoms and analysed for viral DNA.

Sequence analysis. Both strands of DNA were sequenced using the chain termination method (Sanger *et al.*, 1977) by subcloning and by the use of specific primers. Sequence data was analysed using the DNASTAR package (version 1.02, DNASTAR Inc., Madison, Wis., USA) available for the Apple Macintosh computer. The Megalign program available with the same package was used to align ToLCV-India sequences with other geminivirus sequences.

Construction of mutants. Two different mutations were constructed for coat protein (AV1). Clone pMPA1 was linearized at the unique *Sty*I site [at nucleotide (nt) 479, Fig. 2*a*], end-filled with the Klenow fragment of DNA polymerase I and religated to give clone pCPM1. End-filling and religation inserted four nucleotides resulting in a frame shift after amino acid (aa) 65 and termination after aa 69. Another mutation was introduced by deleting the 61 bp *Nsi*I fragment (nt 801–862, Fig. 2*a*) resulting in a frame shift after aa 172 and termination after aa 177 (clone pCPM2).

Mutations were confirmed by sequencing and the loss or gain of restriction enzyme sites where appropriate. Partial head-to-tail dimers made from the mutants were used to infect *N. benthamiana* and tomato plants.

Detection of viral DNA forms in inoculated plants. Total DNA (1 μg) from the upper leaves of *N. benthamiana* or tomato plants (prepared following the procedure of Dellaporta *et al.*, 1983) was separated in a 1% agarose gel and Southern blotted using the standard protocols (Sambrook *et al.*, 1989). A 1·6 kb *Bam*HI–*Eco*RI fragment specific to A component (pMPA1) and which contains ORFs AV1, AV2, AC2 and AC3 was used as the radioactively labelled probe.

Confirmation of mutations in inoculated plants. Stability of mutations in the CP sequence was confirmed by PCR amplification of a 993 nt fragment covering ORFs AV1, AV2 and AV3 from total DNA isolated from inoculated plants and restriction digestion to identify loss or gain of enzyme sites. Total plant DNA (1 ng) was amplified using primers designed to anneal in the common region (nt 1–18 of pMPA1, Fig. 2*a*) and 3′ CP sequence (nt 974–993 of pMPA1, Fig. 2*a*) by 30 cycles of melting, annealing and synthesis conditions of 94 °C for 1 min, 52 °C for 1 min and 72 °C for 2 min. The amplified fragments were purified on QIA quick spin columns (Qiagen) before restriction digestion and electrophoresis in 1·2% agarose gels.

Results

Identification of two distinct A components of ToLCV-India

Cross-hybridization among 25 ToLCV-India clones, initial sequencing of several clones and sequence comparison with other geminiviruses showed that 17 clones

Table 1. *Infectivity of genome components and mutants of ToLCV-India in Nicotiana benthamiana and tomato*

DNA A	DNA B	Infectivity (infected/inoculated)	Symptom type
N. benthamiana			
pMPA1	–	0/10	None
pMPA1	pMPB1	20/20	Severe
pMPA1	pMPB2	10/10	Severe
pMPA2	–	0/10	None
pMPA2	pMPB1	14/20	Mild
pMPA2	pMPB2	8/10	Mild
pMPA1 + pMPA2	–	0/10	None
pMPA1 + pMPA2	pMPB1	10/10	Severe
pMPA12	–	0/10	None
pMPA12	pMPB1	10/10	Severe
pCPM1	pMPB1	10/10	Severe
pCPM2	pMPB1	9/10	Severe
Tomato			
pMPA1	pMPB1	10/10	Severe
pMPA2	pMPB1	9/10	Mild
pCPM1	pMPB1	10/10	Severe

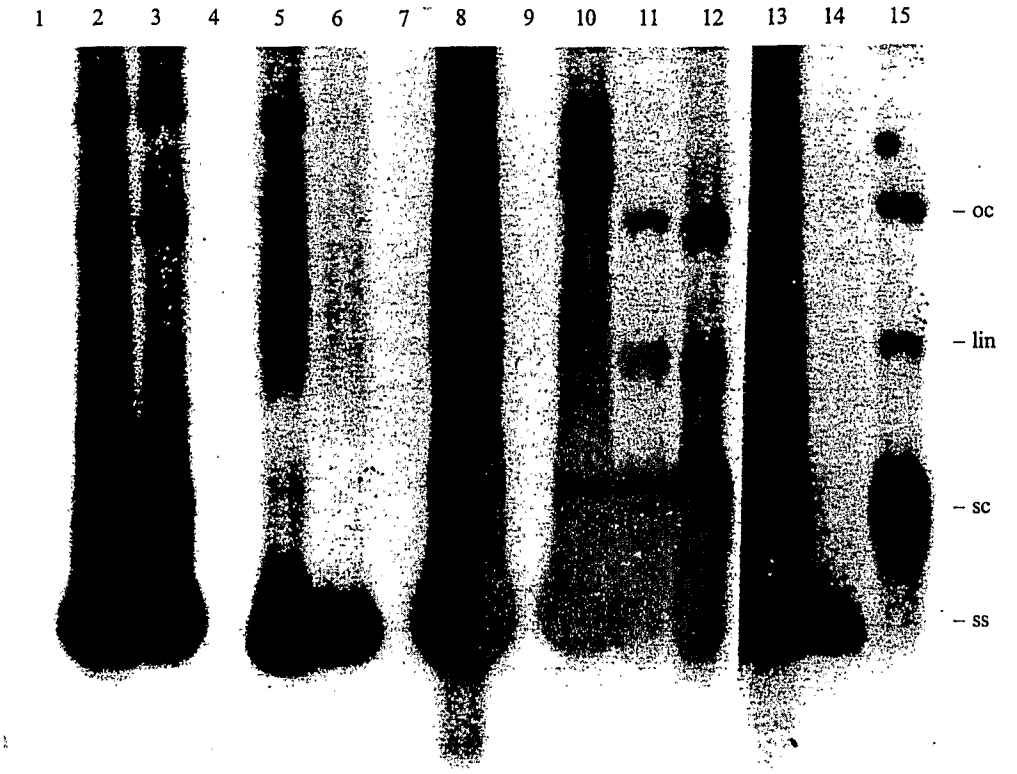

Fig. 1. Southern blot analysis of *N. benthamiana* and tomato plants inoculated with ToLCV-India genome components and mutants. Total DNA (1 μg) extracted from the leaves of inoculated *N. benthamiana* (lanes 1–12) and tomato (lanes 13–15) plants was separated on a 1% agarose gel, blotted to nylon membrane and hybridized with a probe specific to A component. Lane 1 contains DNA extracted from plants inoculated with pMPA1 alone; lane 2 with pMPA1 + pMPB1; lane 3 with pMPA1 + pMPB2; lane 4 with pMPA2 alone; lane 5 with pMPA2 + pMPB1; lane 6 with pMPA2 + pMPB2; lane 7 with pMPA1 + pMPA2; lane 8 with pMPA1 + pMPA2 + pMPB1; lane 9 with pMPA12 alone; lane 10 with pMPA12 + pMPB1; lane 11 with pCPM1 + pMPB1; lane 12 with pCPM2 + pMPB1; lane 13 with pMPA1 + pMPB1; lane 14 with pMPA2 + pMPB1; and lane 15 with pCPM1 + pMPB1. The position of viral single-stranded (ss) and double-stranded supercoiled (sc), linear (lin) and open circular (oc) DNA forms are indicated. The DNA in each lane was extracted from pooled tissues from 10 inoculated plants.

belonged to the A component and eight to the B component (data not shown). All 25 clones came from the same DNA sample that was isolated from young leaves of several infected plants collected from the same area. Restriction mapping revealed that A component clones fell into two types that differed with regards to several enzyme sites. For example, eight clones had additional *Bam*HI and *Hin*dIII sites. Similarly, B component clones also fell into two types. One clone from each type was selected for further analysis; pMPA1 and pMPA2 [cloned at the *Eco*RV site of pBluescript SK(−)] for A component, and pMPB1 [cloned at the *Hin*dIII site of pGEM-7zf(+)] and pMPB2 (cloned at the *Pst*I site of pTZ18R) for B component.

Partial tandem repeats of the selected clones were inoculated onto *N. benthamiana* in all possible combinations (data summarized in Table 1). Clone pMPA1, in combination with pMPB1 or pMPB2, gave severe symptoms. The characteristic downward leaf curling appeared within 5 days of inoculation, and after 3 weeks the plants had interveinal chlorosis and marked reduction in leaf size and internode length. In contrast, pMPA2, either with pMPB1 or pMPB2, gave mild symptoms. The downward curling appeared 2 to 3 days later than for pMPA1 and 3 weeks later the plants had no chlorosis and less pronounced reduction in leaf size and internodal length compared to plants infected with pMPA1. When both pMPA1 and pMPA2 were co-inoculated along with pMPB1, the symptom appearance was of the pMPA1 type. Severe symptoms were also observed when a dimer (pMPA12) containing 0·9 copies of pMPA1 and 1·0 copy of pMPA2 was inoculated along with pMPB1 (Table 1). No symptoms developed in plants inoculated with A component (pMPA1, pMPA2, pMPA1 + pMPA2, or pMPA12) alone (Table 1).

When coinoculated to tomatoes, pMPA1 and pMPB1 induced symptoms typical of the tomato leaf curl disease from India, i.e. dark greening and downward curling of

(*a*)

Fig. 2. For legend see opposite.

the leaves and thickening of the veins strongly suggesting that the cloned DNAs are effectively the causal agents of the disease. The severe and mild symptoms caused by pMPA1 and pMPA2 were also observed when tomato plants were coinoculated with these and with pMPB1, indicating that differences in symptoms induced by pMPA1 and pMPA2 were not limited to *N. benthamiana* (Table 1).

Total leaf DNA prepared from a pool of upper leaves of 10 plants, each inoculated with A component clones alone or combinations of A and B component clones, was analysed by Southern blot hybrization using a probe specific for the A component (Fig. 1). The results showed that both ss- and ds- viral DNA forms were present in plants inoculated with pMPA1 in combination with the B component clones pMPB1 or pMPB2 (Fig. 1, lanes 2, 3 and 13). There was less dsDNA, particularly supercoiled DNA form, in plants inoculated with pMPA2 in combination with the B component clones pMPB1 or pMPB2 (Fig. 1, lanes 5, 6 and 14). No viral DNA was

(*b*)

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                                   -                            G A     G         C
TAATATTACCGAAAGGCCGCGAAAATTTTGACCCCCTTATCCTGACCGTTGATGCGTAATCATTGCA-CGCCGTTATCCGTCC-GATTTGCAACACGTGTAT 100
T                      <
CCCACTAACAGACTTTATGGAAAATAAATGTGTGAATGCGTCTCTTTTCTGCATATGTGTTCCCCATATGTCTTTATCGTACTTCTATTATATGCGTCTG 200
TGGTCCCCCCGCATTATATAAAGTCTTTCACATAAATCAAATTGCCTTCTTTGCTATGTATATTTTGATCGGTCGAGATCAAAATTAATATGTTGCGAAC 300
ATATCCGTCGTTCGATCTTATGAGATATGCTTTAATTCAAACAATACTTGTTTGAATTTTATGCACGCTGTACAATACTAGTTTATAAAACTGCTACATA 400
TGTGACATTACATGGTGTTTCCGTTGCCACACATTTCCTATCCCAGCCAAATGGCGTTTCCCTCTCCTTATTCCACGCCTCGTCGTTCGGGTTACCCATT 500
CAACAGAACATACAACGGAAACAAGAGTTTCCGCTTGTGGAAGACCCGGAAGTATCAAAACTGGAAGCGCTATCGCAGTACCCATTCCATAGCACGTTCT 600
CCAACCGAACTGTTTGGCGATCCAATCTCCAAACAATATACGCGTAAGGAAATCTGTGAAACACAGGAGGGTTCGGAGTATCTGCTGCACAACAATCGTT 700
ACATGACGTCATATGTCACGTATCCATCAAAAACAAGAACTGGAACGGACAAGCGCGTTCGTTCCTATATCAAGCTAAAGAGTCTGAACATATCTGGGAC 800
ATTTGCTGTTCGTAAATCTGACTTGATGACCGAAGTGGTGCAAACAAATGGTCTATACGGAGTGATGTCTATAGTTGTAGTCCGCGATAAATCTCCAAAG 900
ATTTATTCTGCGACCCAACCTTTAATACCGTTTGTTGAGCTATTTGGATCTGTTAATGCTTGCAGGGGCAGTCTGAAAGTGGCAGAACGCCACCATGAAC 1000
GCTTCGTACTTCTGAATCAAACATCCATCGTTGTCAATACCCCACATTCCAACGCTATCAAGAAATTCTGCATTCGTAACTGCATCCCAAGAACTTACAC 1100
AACCTGGGTAACGTTCAAGGACGAAGAAGAAGATAGCTGTACTGGACGATATTCTAACACCCTCCGAAATGCAATTATTTTATATTATGTATGGTTAAGC 1200
GATGTATCCTCACAAGTCGATCTTTACAGCAATGTAATTCTTAATTACATTGGATAATATATAAAAATGCAGAAGAAACATCTTATTTTTTGAATAAATT 1300
TGGCTTAAAATTTATTACACGCTCTTCGATACTGGAGCATTTACATTGGATTTTATACATTGCTCTACAGTCTTCCGTAATTATATCTGCAATCTCTTCC 1400
CTTGTAATACTCCCCGCCTGTGATGCCGATGGACCTGGATCAATTGCCGAATCATCCAATCCGCTCAGATTTTTATATGGTCTGCTGGTGACGGACGAAA 1500
GTCCGATCTCCGATCTGCTTGCCCATGATTCGTTCGGACCTATAGCCAGATAGGGTACCCGTAACGATCTTGAACTATGTCCCATTAACCTTGAACCATC 1600
TACAAGACGCCTTGTTTGTGGTTTGGAACCCACAGACCAGAAATCAATGTCGTTCATAGTGAATTCCTTGGTCTGTATTTCTATCTTTGGTGGTCGGAAT 1700
                                                    >
TCGACGTCAGTCGAATGTTTAGCCGACGACAGCTTCAATTTCCCTAGCATCTTACAGAAGTGTACCCCATTCACGACGTTTGTGTTCTCCACTCGGTATT 1800
                         A
CAACTCTCCAAGGATTCTTATCCTTGAGAGAGAAGAATGAGGAAGAGTAGTAGTGCAGGTTGCAATTGCATTTGATCGGAATTGTGAATTCCGCTTGTTT 1900
TGTGTCCCCCTCCGTCAATCTCATGTCGTGTATCTCTACCACGACATGACCAACAGCATTAATTGGAACCTGACTGCGATATTCCAGAACTACGTGATCT 2000
                               T                  C                              A
ATTTTCATGCATCTATTCCTCAACTGGCTAAGCTTCTGCTCGAACATGGATGGAAATGACAAGGTAACTTCTGCAGCATCGTTTGTGAGAGCGTACTCAA 2100
                                                                              C
CGCGCTCAGATTGAATATACCCACCTACTCCCATACCCATACCATCATTTCCTATTGACATATTGGCCGCGCAGCGCAAAACCCACTGAAACACAGAAGG 2200
       CT           -A                            A                        A     -            T
ACAGACTACGATCAAAGAAACCCCGACGAAGAAGAAACCCTAGCAAACAACGAAGTTGTTTTGCAAAGAACGGATGTAGATGGTTTTATAATGCTATTGC 2300
                       A   T                                          G                          T
ATGTCATGTCTATGTCATACCAATTACCCTAAAATGAACGGCACATATTTTTCTACGAAAAAGGAGTTGTGCATGCATATGGGATGTCTGTTTATTTACG 2400
     T  GA              G                               C                T  T           T
GTATAAATTGGAAGCCCAATTTAT-TTAATTGGGCTGAAGTTTAAATTCAGAAGAAGTCCATGAAATTGGCCCAGCATCCAGGTCCATTGTTAAAATGACA 2500
                         A                                        T
TCGTTTGTGTGTTATTGTGTGTATAGAAGTTAGAGAGAAGCAGCAGTTTCTCTCTCTAGAACTCATCGGGTGTCTCTCAACTTATCTATATAATTGGTGT 2600
                                              T
CTGGAGTCCTATATATAGGTAAGACACCATATGGCATTATTGTAATTGTGAAAAGAAAATTACTTTAATTCAAATTCCCTATAGCGGCCTTTCGTA 2696
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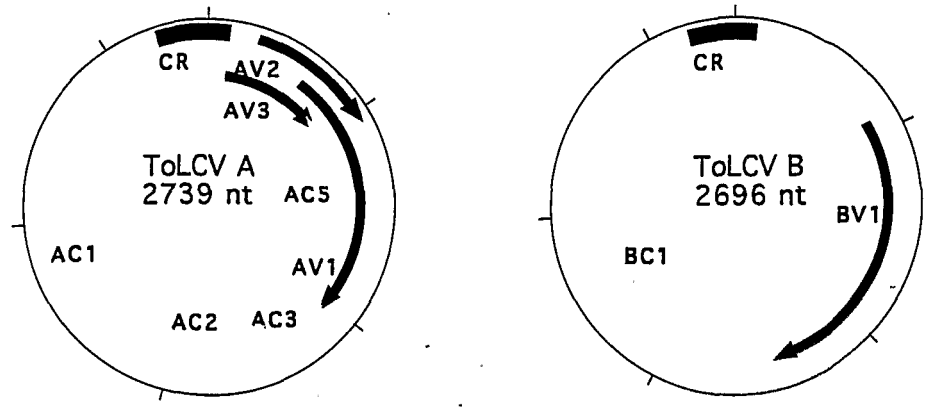
Fig. 2. Nucleotide sequence of the viral sense strands of ToLCV-India DNA components A (*a*) and B (*b*). The lower line in (*a*) is the sequence of pMPA1 and the upper line is of pMPA2 (only the differences from pMPA1 are shown). The upper line in (*b*) is the partial sequence of pMPB2 (nt 1–124 and 1753–2696, > <) and only the differences from pMPB1 (lower line) are shown. Position 1 is the first nucleotide of the invariable nonanucleotide TAATATTAC present in all geminiviruses.

detected in 40 plants inoculated with a components only (Fig. 1, lanes 1, 4, 7 and 9).

Genome organization of ToLCV-India

Complete sequences of clones pMPA1 (A component), pMPA2 (A component) and pMPB1 (B component), and a 1068 nt sequence covering the intergenic region and 5′ region of the ORF BC1 of pMPB2 (B component) were determined in both orientations. Clone pMPB2 was sequenced only partially as its sequenced region was very similar to pMPB1 and plants infected with pMPB1 or pMPB2 (in combination with pMPA1 or pMPA2) developed similar symptoms. The nucleotide sequence of the virion strands of both components are given in Fig. 2. The viral DNAs are circular and the first nucleotide of the nonanucleotide TAATATTAC that is conserved in all geminiviruses sequenced to date is set as nucleotide



ORF	nt	aa	kDa
AV1	287→1057	257	29·6
AV2	127→465	113	13·0
AV3	48→434	129	14·8
AC1	2591←1506	362	40·9
AC2	1603←1184	140	15·7
AC3	1464←1054	137	16·1
AC5	802←317	162	18·0

ORF	nt	aa	kDa
BV1	412→1257	282	32·6
BC2	2161←1283	293	32·9

Fig. 3. Organization of ToLCV-India A (pMPA1, 2739 nt) and B (pMPB1, 2696 nt) components. All ORFs starting with the ATG codon and encoding proteins of larger than 10 kDa are shown as solid arrows. CR represents the 163 nt common region for both components, which contains the 30 nt hairpin loop that is conserved in all geminiviruses. Tables give first and last nucleotides and coding capacity of each ORF.

```
pMPA2  A T T C G G C G T C C T C A A C T T C C T C T A T G T A A T T G G C G T C T G G C G T C C C A T A
pMPA1  A T T C G G C G T C C T C A A C T T A - T C T A T A T G A T T G G T G T C T G G A G T C C T A T A
pMPB1  A T C G G G T G T C T C T C A A C T T A - T C T A T A T A A T T G G T G T C T G G A G T C C T A T A
pMPB2  A T C T G G T G T C T C T C A A C T T A - T C T A T A T A A T T G G T G T C T G G A G T C C T A T A

51     T A T A G G T A G G A C G C T A A A T G G C A A A T T G T A A T T T G G A A A A G A A A A T T A C
50     T A T A G G T A A G A C A C C A T A T G G C A T T A T T G T A A T T T T G A A A A G A A A A T T A C
50     T A T A G G T A A G A C A C C A T A T G G C A T T A T T G T A A T T G T G A A A A G A A A A T T A C
50     T A T A G G T A A G A C A C C A T A T G G C A T T A T T G T A A T T T T G A A A A G A A A A T T A C

101    T T T A A T T C A A A A T C C T C A T A G C G G C C A T T C G T - T A A T A T T A C C G A A T G G C
100    T T T A A T T C A A A T T C C C T A A A G C G G C C A T T C G T A T A A T A T T A C C G A A T G G C
100    T T T A A T T C A A A T T C C C T A T A G C G G C C T T T C G T A T A A T A T T A C C G A A A G G C
100    T T T A A T T C A A A T T C C C T A T A G C G G C C T T T C G T A T A A T A T T A C C G A A A G G C

150    C G C G C A A A T T T T T A
150    C G C G C A A A T T T T T A
150    C G C G A A A A T T T T G A
150    C G C G A A A A T T T T G A
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Fig. 4. Alignment of the nucleotide sequences of the common regions of pMPA1, pMPA2, pMPB1 and pMPB2. The nucleotides that are different from the consensus are boxed.

#1. A (pMPA1; 2739 nt) and B (pMPB1; 2696 nt) component DNAs had little sequence homology except for a 163 nt region that is 93% identical. This region is the characteristic common region (CR) of the bipartite geminiviruses and contains an inverted repeat, which can potentially form a hairpin structure (Lazarowitz, 1992).

There are seven predicted ORFs on the A component and two on the B component with the potential to code for proteins larger than 10 kDa (Fig. 3). We followed the internationally accepted nomenclature for virion (V) and complementary (C) ORFs and analogies with reported geminiviruses while naming the potential coding regions. Six of the nine ORFs shown in Fig. 3 are conserved amongst all bipartite, whitefly-transmitted geminiviruses (WTGs); AV2, AV3 and AC5 are not conserved. ORFs AC1, AC2 and AC3 overlap and are in different reading frames. ORF AC4 (located within AC1 but in a different reading frame), which is conserved in WTGs, is present in ToLCV-India but codes for a small protein of 6·7 kDa. AC5 is located entirely inside the AV1 (the CP gene) but

Table 2. *Nucleotide and amino acid sequence identities* (%) *between TLCV-India* (*pMPA1 and pMPB1*) *and other geminiviruses**

	A	B	IR	AV1	AV2	AC1	AC2	AC3	AC4	BV1	BC1
ACMV-Kenya	60	28	39	73	60	71	53	61	31	23	34
ACMV-Nigeria	61	29	40	74	63	72	55	63	35	24	36
ICMV	62	32	41	90	66	72	55	63	55	36	48
MYMV	55	27	41	76	45	69	–	42	52	23	37
TLCV-Australia	66	–	58	76	54	81	48	60	59	–	–
TYLCV-Egypt	62	–	50	74	61	76	50	64	53	–	–
TYLCV-Israel	63	–	51	73	62	75	50	64	55	–	–
TYLCV-Sicily	63	–	48	74	62	76	49	60	67	–	–
TYLCV-Sardinia	62	–	48	70	61	74	47	56	64	–	–
TYLCV-Thailand[a]	65	28	46	77	66	74	52	64	62	30	44
TYLCV-Thailand[b]	64	28	45	77	63	72	49	66	62	28	48
AbMV	54	27	32	71	–	64	45	41	59	23	31
BDMV	53	29	34	71	–	61	45	41	64	24	38
BGMV-Brazil	53	29	40	74	–	58	45	40	36	23	36
BGMV-Dominican	53	30	35	72	–	63	37	41	67	22	38
BGMV-Guatemala	52	28	33	73	–	61	37	43	64	23	38
BGMV-Puerto Rico	52	29	34	70	–	60	38	43	66	23	38
PHV	53	27	30	73	–	68	40	36	52	26	37
PYMV	54	27	34	72	–	62	46	40	60	22	37
SLCV	46	25	38	73	–	49	40	38	33	24	38
TGMV	54	26	39	71	–	63	42	41	57	23	44
TMoV	54	27	34	72	–	63	43	41	59	23	35
BCTV	35	–	41	12	11	57	22	36	55	–	–
MSV-Kenya	20	–	20	12	17	21	11	–	–	–	–

* A and B are total nucleotide sequences of A and B components, respectively. IR is the ~ 200 nt intergenic region starting from first nucleotide before the start codon of AC1 to the end of the conserved hairpin loop. AV1 to BC1 are conserved ORFs in geminiviruses. The pairwise percentage identities given for A, B and IR are for nucleotide sequences and for AV1 to BC1 are for amino acid sequences. The following sequences were used in comparisons. Whitefly-transmitted, Old World isolates: ACMV-Kenya (Stanley & Gay, 1983), ACMV-Nigeria (Morris *et al.*, 1990), ICMV (Hong *et al.*, 1993*a*), MYMV (Morinaga *et al.*, 1993), ToLCV-Australia (Dry *et al.*, 1993), TYLCV-Egypt (Abdallah *et al.*, 1993), TYLCV-Israel (Navot *et al.*, 1991), TYLCV-Sardinia (Kheyr-Pour *et al.*, 1991), TYLCV-Sicily (G. P. Accotto, personal communication), TYLCV-Thailand[a] (Rochester *et al.*, 1994) and TYLCV-Thailand[b] (S. Attathom, personal communication). Whitefly-transmitted, New World isolates: AbMV (Frischmuth *et al.*, 1990), BDMV (Hidayat *et al.*, 1993), BGMV-Brazil (Gilbertson *et al.*, 1993), BGMV-Dominican Republic (Faria *et al.*, 1994), BGMV-Guatemala (Faria *et al.*, 1994), BGMV-Puerto Rico (Howarth *et al.*, 1985), PHV (Torres-Pacheco *et al.*, 1993), PYMV (Coutts *et al.*, 1991), SLCV (Lazarowitz & Lazdins, 1991), TGMV (Hamilton *et al.*, 1984) and TMoV (Abouzid *et al.*, 1992). Leafhopper-transmitted isolates: BCTV (Stanley *et al.*, 1986) and MSV-Kenya (Howell, 1984).

in the opposite orientation. AV2, which overlaps AV1 in its 3′ end, is found in similar locations only in WTGs isolated from the Old World. AV3 is not reported for any other WTGs, but a smaller ORF in similar position was reported for BCTV. The two ORFs encoded by the B component of ToLCV-India do not overlap (Fig. 3).

Sequence differences between pMPA1 and pMPA2 DNAs of ToLCV-India A component

Since clone pMPA2 caused mild symptoms compared to pMPA1, we carried out a detailed sequence comparison between the clones. Even though both DNAs were of the same length (2739 nt), they had only 94% identity. The differences were due to nucleotide substitutions except for an insertion of nucleotide C at position 2628 and deletion of A at position 2739 of pMPA2 compared to pMPA1 (Fig. 2*a*). One indication of relationship between two sequences of geminiviruses is the identity of nucleotide sequences in the CRs. CR sequences are highly variable, except for a 30 nt conserved stem–loop sequence, compared to other regions of the genome. The CR sequences of both A and B component clones were aligned and are shown in Fig. 4. The CRs of pMPB1 and pMPB2 are 99% identical. The CRs of pMPA1 and pMPA2 were only 80% identical compared to 93% for pMPA1 and pMPB1 and pMPA1 and pMPB2, whereas the CRs of pMPA2 and pMPB1, and pMPA2 and pMPB2 were 77% identical. Amino acid sequence identities between individual ORFs ranged from 91 to 99%, with the greatest degree of similarity in AV1 (CP) with the substitution of Arg for Lys-188 and Val for Ala-254. The AC1 (replication-associated protein) ORFs had 22 differences in amino acids.

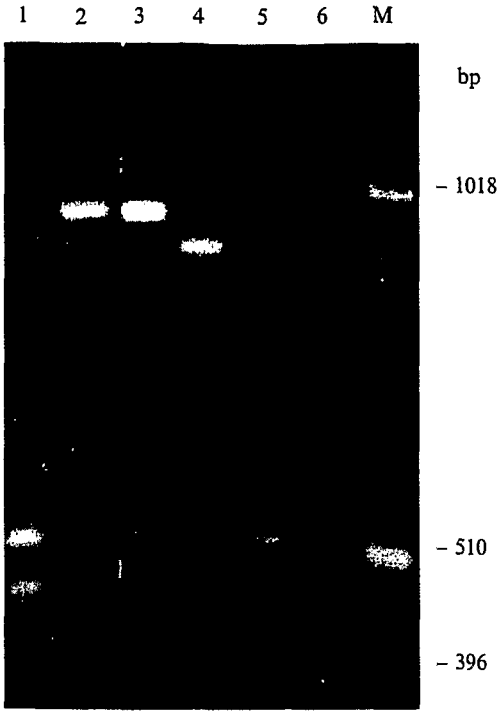

Fig. 5. Analysis of 993 bp PCR amplified fragments from plants inoculated with wild-type and mutant ToLCV-India DNAs. Total DNA (1 ng) extracted from inoculated *N. benthamiana* (lanes 1–4) and tomato (lanes 5 and 6) plants was amplified using primers based on the CR and 3′ terminus of the CP gene as described in Methods. The amplified fragment was purified and digested with *Sty*I (lanes 1, 2, 5 and 6) or left untreated (lanes 3 and 4) before separating on a 1·2% agarose gel. Lanes 1, 3 and 5 contain amplified fragments from DNA extracted from a pool of 10 plants each inoculated with pMPA1, lanes 2 and 6 with pCPM1, and lane 4 with pCPM2. All inoculations included B component DNA pMPB1. The positions of marker fragments (in bp) are shown.

Comparison of ToLCV-India sequences with other geminiviruses

Nucleotide sequences of A component (pMPA1), B component (pMPB1) and the intergenic region of pMPA1, and deduced amino acid sequences of putative products of the ORFs were compared with those of other geminiviruses. Table 2 shows the pairwise percentage identities. ToLCV-India had a total A component sequence identity of 60–66% with WTGs from the Old World compared to 46–54% with WTGs from the New World. No significant identity was detected between ToLCV-India and subgroup I type virus MSV (20%). The percentage identity between BCTV, subgroup II type virus and ToLCV-India was 35%. Among the products of individual ORFs, ToLCV-India AV1 (the CP) showed a high degree of identity with CP from the WTGs (70–90%), with closest identity to Indian cassava mosaic virus (ICMV, Table 2). The product of AC1 also had high sequence identity with the corresponding ORF from the WTGs, but the percentage identity decreased when the New World viruses were compared. When products of AC2 and AC3 served as the basis for comparison, ToLCV-India was closer to viruses from the Old World than to viruses from the New World. For the BV1 and BR1 ORFs, ToLCV-India showed a high degree of similarity to ICMV and the lowest to Abutilon mosaic virus (AbMV).

The predicted protein product of ORF AV3 was compared to ORF V3 located at the equivalent position in BCTV, which was recently shown to be involved in virus movement (Frischmuth *et al.*, 1993; Hormuzdi & Bisaro, 1993). This ORF has not been reported for other geminiviruses and is unusual in that 22 of 128 amino acids in ToLCV-India and 15 of 88 amino acids in BCTV are serine residues. However, the overall percentage identity between the two ORFs was not significant (16%).

The ORF AC4 (located within AC1 but in a different reading frame) of ToLCV-India terminates after aa 59 and is capable of encoding a protein of 6·7 kDa. A similar ORF but with a potential to code for a protein of 11–19 kDa has been reported in the literature only for the WTGs from the Old World and for BCTV. An equivalent ORF is also found in some of the WTGs viruses from the New World (Stanley *et al.*, 1992). Our search for similar ORFs in other geminiviruses showed that it is present in all the WTGs from the New World and is absent from subgroup I geminiviruses. The predicted coding capacity of AC4 in the WTGs from the New World is approximately 9 kDa with the exception of squash leaf curl virus (SLCV) and pepper huasteco virus (PHV), which encode a protein of 14·2 and 6·7 kDa, respectively. The identities between deduced amino acid sequences of AC4 of ToLCV-India and other viruses ranged from 31–67% (Table 2). TYDV, a subgroup I virus but infecting dicotyledonous plants, also has an ORF at a similar position that codes for a protein of 14·3 kDa. The identity between this predicted protein and AC4 of ToLCV-India was only 17% but two short streches of amino acids are conserved (data not shown).

The ORF AC5, located inside the CP gene but in the opposite orientation, has been reported for ICMV (Hong *et al.*, 1993*a*) and PHV (Torres-Pacheco *et al.*, 1993); it is not known if AC5 is transcribed and translated. The deduced amino acid sequence of AC5 ORF of ToLCV-India is 39% and 29% identical to AC5 of ICMV and PHV, respectively.

CP of ToLCV-India is not required for systemic infection and symptom development

A frame-shift mutation was introduced in the CP of ToLCV-India by inserting four nucleotides at the unique *Sty*I site resulting in termination of CP after aa 69 to give

pCPM1. A deletion of a 61 bp *Nsi*I fragment (nt 801–861 of pMPA1) resulted in the termination of CP after aa 177 (pCPM2). The mutant DNAs were coinoculated with wild-type B component DNA pMPB1. The mutants were highly infectious when inoculated onto *N. benthamiana* (Table 1) and there was no delay in symptom appearance. However, the symptoms were different from those in plants inoculated with wild-type pMPA1. The plants had characteristic downward leaf curling but lacked chlorosis and had larger leaves compared to plants inoculated with wild-type virus. Tomato plants inoculated with pCPM1 also showed different symptoms compared to plants inoculated with wild-type pMPA1. Southern blot hybridization analysis showed that inoculated plants had markedly reduced amounts of viral ssDNA (Fig. 1, lanes 11, 12 and 15).

The mutations in CP were maintained in inoculated plants as demonstrated by restriction enzyme analysis of PCR amplified fragments. The primers selected were complementary to A component pMPA1 nt 1–18 and nt 974–993. Restriction analysis of the 993 bp PCR amplified fragment confirmed that the mutations were retained; amplified fragment from pCPM1 inoculated plants had no *Sty*I site, and the fragment from pCPM2 inoculated plants was, as expected, 61 bp shorter than wild-type DNA (Fig. 5).

Discussion

We determined the complete nucleotide sequence of two infectious isolates of ToLCV-India. The genome organization of ToLCV-India is typical for WTGs with bipartite genomes. It contains nine of the ORFs (AV1, AV2, AC1, AC2, AC3, AC4, AC5, BV1 and BV2) reported for WTGs but the ORF AC4 is short compared to those in other WTGs. Disruption of AC4 in ACMV and TGMV had no effect on infectivity or symptom development in *N. benthamiana* plants (Elmer *et al.*, 1988; Etessami *et al.*, 1991). However, in *N. benthamiana* protoplasts expression of ORF AC4 from a plasmid resulted in the supression of expression from the AC1 promoter on another plasmid (Groning *et al.*, 1994). The novel feature in the ToLCV-India genome is the presence of the AV3 ORF, which overlaps ORFs AV1 and AV2. A smaller ORF V3 at the same position was reported for BCTV and is involved in virus movement (Frischmuth *et al.*, 1993; Hormuzdi & Bisaro, 1993). The unusual feature of the predicted AV3 gene product is the high content of serine residues (22 out of 128 amino acids) and highly hydrophilic nature. It is interesting to speculate that ToLCV-India AV3 may have a function similar to V3 in BCTV.

An unexpected result of our study was the cloning of two distinct, but homologous, A components of ToLCV-India (pMPA1 and pMPA2). The diseased tomato plants from which viral DNA was isolated were collected from a 100 m^2 plot in New Delhi, India. All the 25 clones came from a DNA sample prepared from a pool of young leaves and branches of several diseased plants, and not from a single plant. Mixed infections in the field are not uncommon and two strains of squash leaf curl virus (SLCV) that differed in host range have previously been cloned from a field sample (Lazarowitz, 1991). The CRs of the two cloned ToLCV-India B components (pMPB1 and pMPB2) are 99% identical and are more similar to the CR of pMPA1 than to the CR of pMPA2, which may suggest that pMPA1 and pMPB1 and pMPB2 are components of a single strain. Also, pMPA1 in combination with pMPB1 or pMPB2 gave severe symptoms. The differences in CRs of pMPA2 and pMPB1 may result in less efficient replication of pMPB1, resulting in mild symptoms. On the other hand, we cannot rule out the possibility that pMPA2 is a satellite DNA that uses pMPA1 for propagation. It is also possible that there is another B component corresponding to pMPA2 that we have not yet cloned.

The experiments with CP mutants clearly indicate that ToLCV-India is capable of replication, spread and symptom development in the absence of CP. Inoculated plants had attenuated symptoms and produced markedly reduced amounts of ssDNA. This is in contrast to similar experiments reported for TGMV (Gardiner *et al.*, 1988). Plants inoculated with TGMV CP mutants contained both ds- and ss- viral DNAs in the same relative proportions as in wild-type TGMV infections, suggesting that encapsidation is not necessary for accumulation of TGMV ssDNA. In the case of ACMV, plants inoculated with CP mutants had reduced amounts of ssDNA (Stanley & Townsend, 1986). In contrast, the requirement of CP for spread and symptom development is a common feature in monopartite whitefly-transmitted and leafhopper-transmitted geminiviruses (Boulton *et al.*, 1989; Briddon *et al.*, 1989; Lazarowitz *et al.*, 1989; Rigden *et al.*, 1993; Woolston *et al.*, 1989). The features of CP mutants of TYLCV-Thailand are intermediate between those of monopartite and bipartite viruses (Rochester *et al.*, 1994). Mutations in TYLCV-Thailand CP resulted in the delay of symptom induction and decrease in severity of symptoms and amounts of ssDNA. It appears that the CP has different functions in different viruses.

Comparison of the complete nucleotide sequences and predicted amino acid sequences of each ORF showed that ToLCV-India is related to the WTGs from the Old World (Table 2). Interestingly, among the products predicted by the ORFs, the CP of ToLCV-India is more closely related to that of ICMV (90%) than to CP of other viruses. Furthermore, the replication-associated

protein (AC1) is more closely related to that of ToLCV-Australia (81%) than to other replication-associated proteins, suggesting that a recombination event may have occurred during evolution of ToLCV-India. Evidence for a possible recombination event has been presented for BCTV; this virus has a genome organization similar to that of WTGs, and a CP sequence similar to the leafhopper-transmitted geminiviruses (Briddon *et al.*, 1990). An alternative explanation is that these viruses share a common ancestor and have diverged in response to vector biotypes and host species. Matthews (1991) proposed that CP sequences evolve in response to vectors, while replication-associated proteins evolve in response to the host. Recent comparison of deduced amino acid sequences of the CPs of many tomato (yellow) leaf curl isolates indicated that different lineages have evolved in different geographical regions but the variation within a region is limited (Hong *et al.*, 1993*b*). The remarkable similarity between CP sequences of ToLCV-India and ICMV could be because the same whitefly biotype transmits both viruses. It is not known if more than one whitefly biotype exists in India.

ToLCV-India sequences are sufficiently different from those of other viruses (including ToLCV from Australia) to consider it a distinct virus, rather than a strain of another virus. Direct sequence comparisons of geminivirus sequences suggests that strains of the same virus have greater than 90% identity throughout the viral genome (unpublished results). The sequence of the A component of ToLCV-India has only 46–66% sequence identity with the genomes of other WTGs. It also differs from other geminiviruses of the Old World that infect tomato in regard to the requirement for infection and symptom development of the B component; TYLCV-Egypt (Abdallah *et al.*, 1993), TYLCV-Israel (Navot *et al.*, 1991), TYLCV-Sicily (G. P. Accotto, personal communication), TYLCV-Sardinia (Kheyr-Pour *et al.*, 1991) and ToLCV-Australia (Dry *et al.*, 1993) do not have a B component while TYLCV-Thailand (Rochester *et al.*, 1990) has a B component that is not required for infection. ToLCV-India readily infects *N. tabacum*, unlike ToLCV-Australia and TYLCV-Thailand. Another line of evidence for considering ToLCV-India to be a distinct virus is the non-infectious nature of pseudorecombinants between the ToLCV-India A component and TYLCV-Thailand B component (unpublished results). The ability to generate pseudorecombinants between DNA components of bipartite viruses has been suggested as one of the criteria to identify strains of the geminiviruses (Stanley, 1991). If the proposal that bipartite geminiviruses evolved from monopartite geminiviruses (Howarth & Goodman, 1986; Kikuno *et al.*, 1984) is valid, ToLCV-India could be seen as having evolved more recently compared to other geminiviruses that infect tomato in the Old World. The requirement for the B component and dispensability of CP of ToLCV-India for symptom development places it closer to ACMV than to geminiviruses that infect tomato. It is not known if ICMV requires a B component and CP for symptom development. If it does, ToLCV-India and ICMV would appear to be closely related viruses that adapted to different host plants in the same geographical region. The unique features of ToLCV-India make it an interesting virus to study virus spread and replication in tomatoes.

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