VIROLOGY 224, 390–404 (1996)
ARTICLE NO. 0546

The Role of AV2 (“Precoat”) and Coat Protein in Viral Replication and Movement in Tomato Leaf Curl Geminivirus

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Received May 20, 1996; accepted August 16, 1996

We analyzed various mutants of tomato leaf curl virus-India to investigate the role of ORFs AV3, AV2, and coat protein (CP) in viral replication, movement, and symptom development. The results of these studies indicate that ORF AV3 does not encode a protein. Plants inoculated with infectious DNA which contained deletions in AV2 developed very mild symptoms and accumulated only low levels of both single-stranded (ss) and double-stranded (ds) viral DNA, whereas inoculated protoplasts accumulated both ss and dsDNA to wild-type levels, showing that AV2 is required for efficient viral movement. However, both plants and protoplasts inoculated with substitution, frameshift, and other similar mutations in AV2 accumulated low levels of viral DNA. The low levels of accumulation of DNA of these mutants were apparently not due to a defect in AV2 synthesis. Mutations in the CP caused a marked decrease in ssDNA accumulation in plants and protoplasts while increasing dsDNA accumulation in protoplasts. Mutations in both AV2 and CP behaved like AV2 mutants in plants and like CP mutants in protoplasts. The results demonstrated that multiple functions provided by AV2, BV1, and BC1 are essential for viral movement, and that changes in A-component virion-sense mRNA structure or translation affect viral replication.

INTRODUCTION

The geminiviruses are a group of plant infectious agents having circular, single-stranded (ss) DNA genomes packaged within geminate particles. They are transmitted by whitefly and leafhopper vectors and cause significant damage to crop plants (Goodman, 1981; Lazarowitz, 1992; Stanley, 1985; Timmermans *et al.,* 1994). The majority of whitefly-transmitted geminiviruses (WTGs, subgroup III) have bipartite genomes (A and B components). There is little sequence homology between A and B components except for a 200-bp “common region” which contains promoter elements and sequence elements required for DNA replication (Eagle *et al.,* 1994; Laufs *et al.,* 1995; Zhan *et al.,* 1991). Component A encodes all the viral information necessary for the replication and encapsidation of both DNAs (Rogers *et al.,* 1986; Sunter *et al.,* 1987; Townsend *et al.,* 1986). The B component encodes two proteins that are necessary for efficient systemic spread of the virus in plants (Brough *et al.,* 1988; Etessami *et al.,* 1988; Ingham *et al.,* 1995; von Arnim *et al.,* 1993).

Many different WTGs infect tomato in different parts of the world. Tomato golden mosaic virus (TGMV; Hamilton *et al.,* 1984), tomato leaf crumple virus (TLCrV; Paplomatas *et al.,* 1994), and tomato leaf curl virus (ToLCV)-India (Padidam *et al.,* 1995a) have B components that are essential for systemic infection and symptom development, whereas a B component has not been isolated for ToLCV-Australia (Dry *et al.,* 1993), tomato yellow leaf curl virus (TYLCV)-Israel (Navot *et al.,* 1991), or TYLCV-Sardinia (Kheyr-Pour *et al.,* 1991). The B component has been isolated for TYLCV-Thailand but is not required for infection (Rochester *et al.,* 1990). These viruses differ in the requirement of coat protein (CP) for viral spread and symptom development. While CP is essential for spread and symptom development in ToLCV-Australia (Rigden *et al.,* 1993), mutations in CP delay and reduce symptom development in TYLCV-Thailand (Rochester *et al.,* 1994) and TGMV (Gardiner *et al.,* 1988).

The tomato-infecting viruses also differ in the number of open reading frames (ORFs) on the A component. Viruses infecting tomato in the old world (ToLCV and TYLCV isolates) possess two virion-sense overlapping ORFs (CP and AV2) while viruses infecting tomato in the new world (TGMV and TLCrV) have a single ORF, encoding the CP. The association of WTGs having different biological properties with tomato provides an opportunity to study host–viral interactions in disease development.

We are studying the functions of the A-component virion-sense ORFs of ToLCV-India in virus replication, movement, and symptom development. Unlike other WTGs, ToLCV-India has three A-component virion-sense

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0042-6822/96 $18.00

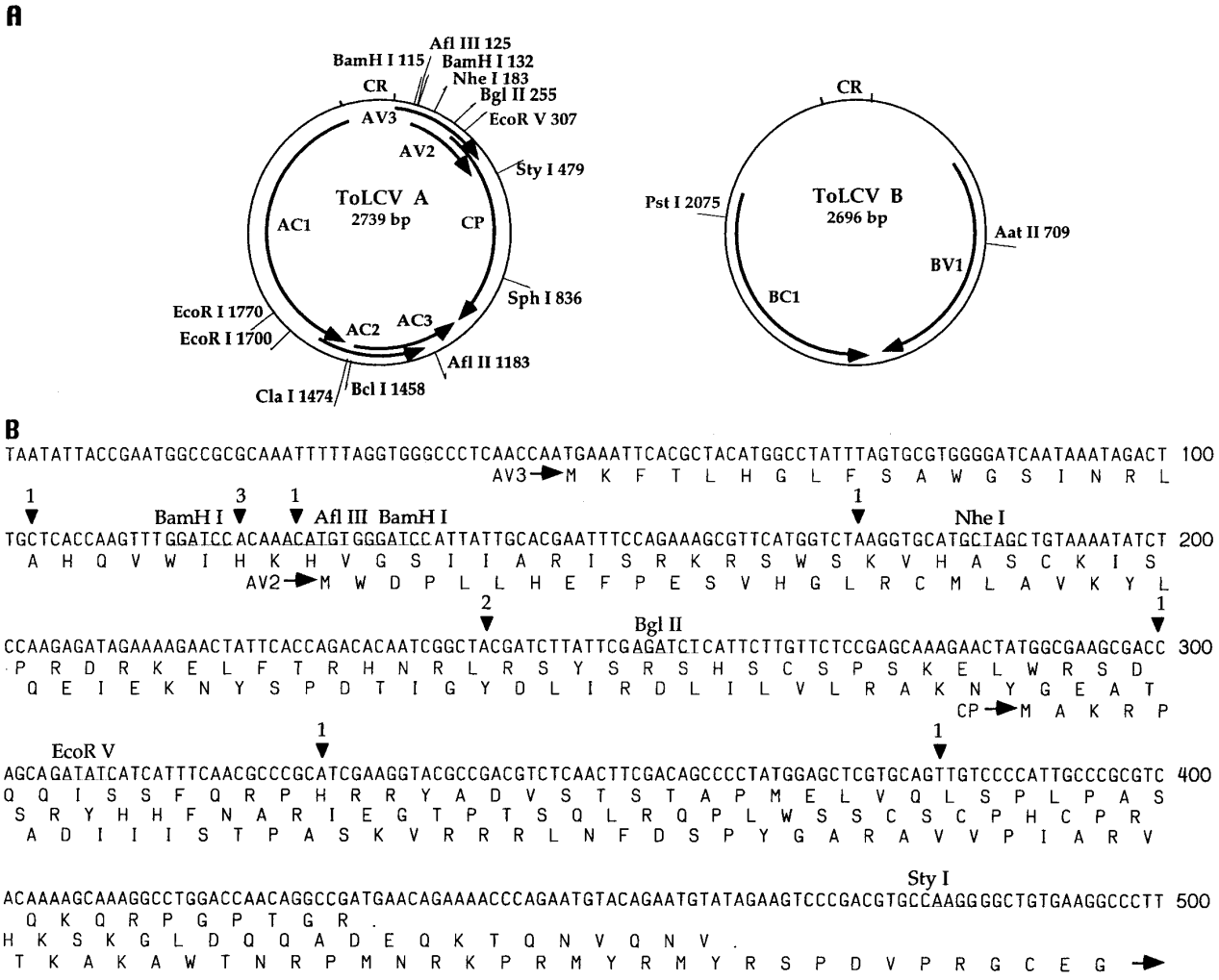

FIG. 1. Genome organization and nucleotide sequence of overlapping ORFs AV3, AV2, and CP of ToLCV-India. (A) Genome organization of ToLCV-India showing the position of virion-sense and complementary-sense ORFs and the common region (CR). Restriction sites relevant to this study are shown. (B) Virion-strand sequence at the overlapping region of ORFs AV3, AV2, and CP from the A-component clone pMPA1 (Padidam *et al.,* 1995a). The predicted amino acid sequences are shown below nucleotide sequence. The 5′ ends of the RNAs determined by the 5′ RACE PCR are identified with filled triangles and the numbers of independent clones at each coordinate are indicated above the nucleotide. Restriction sites are underlined.

ORFs, namely, CP, AV2, and AV3 (Padidam *et al.,* 1995a). We reported previously that mutations in CP did not affect systemic movement and symptom development by the virus (Padidam *et al.,* 1995a). We show here that ORF AV3 of ToLCV-India does not encode a protein, that AV2 is required for efficient viral movement, and that changes in sequence of the overlapping AV2 and CP genes, or their translation, affect viral replication.

MATERIALS AND METHODS

Construction of mutants and gene expression cassettes

The A (pMPA1) and B (pMPB1) components of ToLCV-India used in this study have been described previously (Padidam *et al.,* 1995a) and the arrangement of their genes is shown in Fig. 1A. The nucleotide (nt) sequence and the predicted amino acid (aa) sequence of the overlapping region of ORFs AV3, AV2, and CP are shown in Fig. 1B.

The descriptions of the mutants and the expression cassettes are summarized in Table 1. Oligonucleotide-mediated mutagenesis was performed by using the Transformer mutagenesis kit available from Clonetech Laboratories. The kit is based on the method of Deng and Nickoloff (1992) and uses double-stranded (ds) DNA as template. Mutagenic oligonucleotides were designed to substitute termination or missense codons in place of codons for amino acids. Frame-shift mutations were introduced at available restriction sites by filling in 5′ overhangs or deleting the 3′ overhangs by T4 DNA polymerase and religating the plasmids. Deletions were made by taking advantage of the restriction sites flanking the sequences to be deleted.

TABLE 1

Description and Method of Construction of Mutants and Expression Cassettes Used in This Study

Construct	Description and method of construction
	Mutations in AV3
M1R	Met1 codon changed to Arg codon by mutating T to G at nt position 49 by oligonucleotide-directed mutagenesis. This created a *Sty*I site.
I24V;H25D	Ile24 and His25 codons changed to Val and Asp codons, respectively, by mutating A to G at nt position 117 and C to G at nt position 120 by oligonucleotide-directed mutagenesis. This resulted in the conversion of the first *Bam*HI site to *Sal*I site.
K26te	Lys26 codon changed to termination codon by mutating A to T at nt position 123 by oligonucleotide-directed mutagenesis.
	Mutations in AV2
M1te	Met1 codon changed to termination codon by replacing the 17-bp *Bam*HI fragment (nt 115 to 137) with a 17-bp synthetic double-stranded oligonucleotide that had AT to TA mutation at nt position 127 and 128. This resulted in loss of the unique *Afl*III site and gain of a *Spe*I site.
M19T	Met19 codon changed to Thr by mutating nt TG to CA at positiosn 182 and 183 by oligonucleotide-directed mutagenesis. This resulted in the loss of the unique *Nhe*I site.
M1te;M19T	Met1 codon and Me19 codon changed to termination codon and Thr codon, respectively. Constructed by replacing the *Bam*HI fragment (nt 115 to 137) of the mutant M19T with the synthetic double-stranded oligonucleotide as in the M1te mutant.
In21S	Insertion of Ser after Leu20. Nucleotides AGT were inserted at position 187 by partial end-filling and religating at the unique *Nhe*I site. The mutation resulted in the loss of *Nhe*I site.
Y24te	Tyr24 codon changed to termination codon by mutating TC to AG at nt positions 198 and 199 by oligonucleotide-directed mutagenesis.
C84S;C86S	Cys84 and Cys86 codons changed to Ser and Ser codons, respectively, by mutating nt T to A at positions 376 and 382 by oligonucleotide-directed mutagenesis. The mutation created a *Xho*I site at nt position 373.
Q104te	Gln104 codon changed to termination codon by a tranversion of C to T at nt position 436 by oligonucleotide-directed mutagenesis.
Δ121-137	Deletion of the 17-bp *Bam*HI fragment, made by digesting with *Bam*HI and religation. This deleted nt 121–137 coding for Met1 and Trp2 and 6 bp before Met1 codon.
Δ121-137;M19T	Deletion of the 17-bp *Bam*HI fragment and Met19 codon changed to Thr codon. Created by digesting the mutant M19T DNA with *Bam*HI and religating.
Δ121-137;Y24te	Deletion of the 17-bp *Bam*HI fragment and Tyr24 codon changed to termination codon. Created by digesting the mutant Y24te with *Bam*HI and religating.
Δ121-184	Deletion of the 64-bp fragment between the first *Bam*HI and *Nhe*I sites. Made by digesting with *Bam*HI and *Nhe*I, end-filling and religation. This resulted in the deletion of 7-bp before the Met1 codon and the sequence coding for Met1 to Met19.
Δ127-286	AV2 deletion mutant that codes for CP positioned at the AV2 Met1 site. Nucleotides 127 to 286 were deleted by cloning a PCR-amplified CP (nt 287 to unique *Afl*II site at nt 1182; amplified by 10 cycles with 1.0 μg DNA template) between *Afl*III (nt 127) and *Afl*II sites (nt 1182).
Inv121-131	Met1 and Tyr2 codons changed to Phe and Val codons, respectively, by replacing the 17-bp *Bam*HI fragment with the 17-bp synthetic double-stranded oligonucleotide of the mutant M1te, but in reverse orientation. This also changed the 6-nt sequence before Met1 codon.
Δ121-126	Deletion of the nt -6 to -1 of the AV2 ORF (nt 121 to 126). Constructed by PCR amplifying the AV2 5′ fragment [nt 127–183 (first ATG to *Nhe*I) amplified by 10 cycles wtih 1.0 μg DNA template] with a *Bam*HI site before the ATG and inserting this fragment between the first *Bam*HI and *Nhe*I site.
	Mutations in CP
R66fr	End-filling and religation at the unique *Sty*I site at position 479. This resulted in the insertion of 4 bp causing a frame shift at Arg66 codon and termination after aa 69.
C68S;C72S	Cys68 and Cys72 codons changed to Ser and Ser codons, respectively. Nucleotides G at position 489 and T at position 500 were changed to C and A, respectively, by oligonucleotide-directed mutagenesis.
H85K	His85 codon changed to Lys codon by mutating nt C to A and nt T to G at positions 539 and 541, respectively, by oligonucleotide-directed mutagenesis.
T251te	Tyr251 codon was changed to a termination codon by mutating nt T to A at position 1039 and nt A to C at position 1041 by oligonucleotide-directed mutagenesis. This created a *Hin*DIII site and resulted in the truncation of CP 6 aa prematurely.
	Mutations in both AV2 and CP
M1te/R66fr	A double mutant of AV2-M1te and CP-R66fr, constructed by end-filling and religating at the *Sty*I site of the AV2-M1te mutant DNA.
M19T/R66fr	A double mutant of AV2 M19T and CP R66fr, constructed by end-filling and religating at the *Sty*I site of the AV2-M19T mutant DNA.

TABLE 1—*Continued*

Construct	Description and method of construction
Δ121-137/R66fr	A double mutant of AV2-Δ121-137 and CP-R66fr, constructed by deleting the 17-bp *Bam*HI fragment of the CP-R66fr mutant DNA.
Y61te/S11te	Termination of AV2 and CP at aa 61 and 11, respectively. Constructed by inserting a 16-bp synthetic palindromic oligonucleotide (CTTAAGTTAACTTAAG) that has termination codons in all three reading frames at the unique *Eco*RV site (nt 308). The oligo has sites for *Afl*II and *Hpa*I.
ΔAV2CP-GFP	A 713-bp sequence coding for green fluorescent protein (GFP, Chalfie *et al.,* 1994) was cloned between end-filled first *Bam*HI site (nt 119) and blunt-ended *Sph*I (nt 836) site. This resulted in the replacement of 716-bp sequence coding for the entire AV2 and aa 1–180 of CP with the sequence coding for the GFP.
	Mutations in other ORFs
AC1M	Rep protein (AC1) truncated after aa 299. Constructed by deleting the 70-bp *Eco*RI fragment (nt 1700 to 1770).
AC2M	A frame-shift mutation of AC2 ORF created by end-filling and religation at the *Cla*I site (nt 1476). Insertion of 2 bp due to end-filling caused frame shift after aa 43 and fused AC2 to the sequence coding the AC3. Expected to code for a protein of 184 aa length.
AC3M	A frame-shift mutation of AC3 ORF created by end-filling and religation at the unique *Afl*II site (nt 1282). Insertion of 4 bp due to end-filling caused frame shift after aa 93 and termination after aa 94.
AC23M	A double mutant of AC2 and AC3 ORFs created by end-filling and religation at the unique *Bcl*I site (nt 1458). The 4-bp insertin due to end-filling resulted in frame shift after aa 48 and termination after aa 81 for AC2 and frame shift after aa 2 and termination after aa 94 for AC3. Frame shift in ORF AC3 created an ORF that can potentially encode a fusion protein comprising first 2 aa of AC3 and aa 47 to end of the AC2.
BV1M	A frame-shift mutation of BV1 created by deleting the 3′ overhang and religating at the *Aat*II site (nt 709). Deletion of 4 bp caused frame shift after aa 88 and termination after aa 120.
BC1M	A frame-shift mutation of BC1 created by deleting the 3′ overhang and religating at the *Pst*I site (nt 2075). Deletion of 4 bp caused frame shift after aa 29 and termination after aa 45.
	Expression cassettes
35S-AV2	A pUC19 plasmid containing an AV2 expression cassette. AV2 ORF was PCR amplified (10 cycles with 1.0 μg DNA template) and cloned between duplicated 35S promoter and NOS terminator sequences. The AV2 ORF sequence was confirmed by sequencing.
35S-CP	A pUC19 plasmid containing a CP expression cassette. CP ORF (*Bgl*II to *Afl*II; nt 255–1183) was cloned between duplicated 35S promoter and NOS terminator sequences.
35S-AV2CP	A pUC19 plasmid containing an AV2 and CP expression cassette. AV2 and CP ORFs (*Afl*III to *Afl*II; nt 125–1183) were cloned between duplicated 35S promoter and NOS terminator sequences.
35S-AV2^{-}CP^{-}	A pUC19 plasmid containing mutated AV2 and CP expression cassette. *Spe*I to *Afl*II (nt 125–1183) fragment of AV2 mutant M1te;M19T was first cloned between duplicated 35S promoter and NOS terminator sequences. CP of this construct was then mutated by end-filling and religation at the unique *Sty*I site (at position 479) to have AV2-M1te;M19T/CP-R66fr DNA under the control of 35S promoter.

Mutations were confirmed by DNA sequence analysis and loss or gain of restriction enzyme site where appropriate. In the case of mutations made by oligonucleotide-mediated mutagenesis, a small restriction fragment encompassing the mutation was recloned into an unmutagenized A component in place of the cognate fragment to avoid the possibility of second site mutations. Partial head-to-tail dimers made from the mutants were used to infect *Nicotiana benthamiana* and tomato (*Lycopersicon esculentum*) plants, and *N. tabacum* BY-2 protoplasts.

Plant and protoplast inoculations

Two-week-old seedlings of *N. benthamiana* and tomato grown in Magenta boxes were inoculated with the head-to-tail partial dimers of the genome using a Bio-Rad helium-driven particle accelerator (Padidam *et al.,* 1995a). Five to twenty plants were inoculated with each mutant (0.5 μg each of A and B component DNA per plant). Plants were scored for symptoms, and the newly formed upper leaves were collected for Southern and Western blot analyses 22–25 days following inoculation.

Protoplasts isolated from BY2 suspension cells were transfected with viral DNAs essentially as described by Watanabe *et al.* (1987). Initial experiments were conducted to optimize the amount of viral DNA and electroporation conditions. One million protoplasts were inoculated by electroporation (250 V, 500 μF) with 2 μg each of A and B component DNAs and 40 μg of sheared herring sperm DNA. Protoplasts were collected from cultures 48 hr postinoculation for DNA isolation and Western blot analysis.

Southern blot analysis

DNA was prepared from systemically infected leaves following the procedure of Dellaporta *et al.* (1983) and

DNA was isolated from protoplasts according to the procedure of Mettler (1987). Total DNA was electrophoresed in 1% agarose gels (without ethidium bromide) and transferred to nylon membranes and viral DNA was detected using an A-component-specific radioactive probe (the 900-bp *Afl*II–*Pst*I fragment containing ORFs AC1, AC2, and AC3) or a probe specific for B component (an 878-bp PCR-amplified fragment containing ORF BC1). The amount of viral DNA was quantitated by exposing the Southern blots to storage phosphor screen plates and counting on a phosphorimager (Molecular Dynamics). The ss viral DNA form was confirmed by its susceptibility to S1 and mungbean nucleases and resistance to exonuclease.

Western blot analysis

Rabbit polyclonal antibodies were raised against the AV2 protein expressed in *Escherichia coli.* The AV2 gene was cloned into the expression vector pTrcHis (Invitrogen) behind the *Trc* promoter and a sequence that encodes a 31-aa peptide that contains 6 tandem His residues. The fusion protein was purified on a nickel column as suggested by the manufacturer (Invitrogen). Rabbit polyclonal antibodies were also raised against synthetic peptides corresponding to coat protein aa 17–30 and 149–164. In addition, polyclonal antibodies raised against purified TYLCV-Sardinia (kindly provided by B. Gronenborn, CNRS, Gif-sur-yvette) were used for selected experiments.

Whole cell protein extracts were prepared in Tris-buffered saline (0.05 *M* Tris–HCl, pH 7.0, 0.15 *M* NaCl). The protein extracts were then fractionated by SDS–PAGE (13%) and transferred to Immobilon-P membranes (Millipore), and CP and AV2 were immunologically detected using ECL–Western blot reagents (Amersham). The emitted light was captured on Hyperfilm-ECL (Amersham) and protein was quantitated using a scanner and the NIH Image computer program.

5′ RACE PCR

Poly(A)$^+$ RNA was prepared from systemically infected leaves of plants inoculated with wild-type viral DNA 22 days postinoculation using Dynabeads coated with oligo(dT)$_{25}$ (Dynal). 5′ ends of transcripts derived from the virion strand of the A component were mapped utilizing a kit for rapid amplification of cDNA ends (5′ RACE) available from Gibco BRL. First-strand cDNA was synthesized using gene-specific primer 1 (comprising nt 414–434 of the complementary sense) and was dC tailed. The dC-tailed cDNA was amplified by PCR using an anchor primer that anneals to the dC tail and gene-specific primer 2 (nt 396–413, complementary sense). Amplified fragments were cloned into the pAMP1 vector (Gibco BRL) utilizing the UDG cloning procedure. The clones were analyzed for insert length, and 11 clones were sequenced using an ABI automated sequencer.

RESULTS

Mutations in AV3 do not affect symptom development and viral DNA accumulation

The predicted ORF AV3, which overlaps the ORFs of AV2 and CP at the 3′ region, encodes a predicted protein of 14.8 kDa and has an unusually high number of Ser residues (Fig. 1). Three mutations were introduced into the 5′ region of the ORF AV3 without affecting the coding sequence of the overlapping ORF AV2 (Table 1). In mutation M1R, the potential to encode the AV3 protein was abolished by mutating the initiation codon to AGG. The substitution mutation I24V;H25D, on the other hand, retained the potential to encode full-length protein. In mutation K26te a termination codon was created in place of a Lys residue.

The infectivity of virus that carried mutations in AV3 was analyzed by coinoculating the mutant A component with the wild-type (wt) B component DNA. All *N. benthamiana* plants inoculated with mutants M1R and I24V;H25D developed severe symptoms identical to plants inoculated with the wt viral DNA (Table 2). The plants were highly stunted with drastic reduction in internodal length and showed reduction in leaf size, pronounced interveinal chlorosis, and curling of leaves. All 10 plants inoculated with the mutant K26te showed severe symptoms but without drastic reduction in internodal length. Tomato plants inoculated with the mutant M1R also showed severe symptoms similar to the plants inoculated with wt DNA.

The accumulation of viral ssDNA and dsDNA in plants infected with the mutants was quantitated (as described under Materials and Methods) and found to be comparable to levels present in plants infected with wt DNA (Fig. 2A, lanes 1–4; Table 2). Restriction enzyme digestion followed by Southern blot hybridization analysis of viral DNA from infected plants showed the pattern expected for mutant DNAs indicating that the infectivity of AV3 mutants was not due to reversion to wild type (data not shown). For example, a *Sty*I site that was created by the M1R mutation was retained in viral DNA isolated from the infected plants.

Protoplasts derived from *N. tabacum* BY-2 suspension cell cultures were inoculated with the AV3 mutants to evaluate the effect of AV3 mutations on viral DNA replication. Protoplasts inoculated with the mutant I24V;H25D accumulated both ss and dsDNA to levels observed in protoplasts inoculated with the wt DNA. The AV3 mutant K26te in contrast accumulated low levels of both ss and dsDNA while the AV3 mutant M1R accumulated low levels of only ssDNA compared to protoplasts inoculated with wt DNA (Fig. 2B, lanes 1–4; Table 2).

TABLE 2

Summary of Results Obtained with the Mutants of ToLCV-India in *N. benthamiana,* Tomato Plants, and *N. tabacum* Protoplasts

Mutant	No. of plants infected/ inoculated	Symptom severity	Plant inoculations[a] DNA[b] ssDNA		Plant inoculations[a] DNA[b] dsDNA		Plant inoculations[a] Protein[c] CP	Plant inoculations[a] Protein[c] AV2	Protoplast inoculations[a] DNA[d] ssDNA		Protoplast inoculations[a] DNA[d] dsDNA		Protoplast inoculations[a] Protein[d] CP		Protoplast inoculations[a] Protein[d] AV2	
	N. benthamiana								*N. tabacum*							
Wild type	30/30	Severe	100	(91–146)	100	(84–134)	100	100	100	(72–127)	100	(78–106)	100	(90–110)	100	(61–139)
AV3 mutants																
M1R	10/10	Severe	73		130		101	60	42	(16–60)	85	(53–121)	60	(40–83)	20	(15–23)
I24V;H25D	10/10	Severe	111		126		103	98	94	(33–150)	85	(66–112)	79	(50–98)	84	(36–138)
K26te	10/10	Severe[e]	103		105		99	10	32	(14–57)	16	(6.7–32)	52	(13–75)	10	(0–16)
AV2 mutants																
M1te	20/20	Very mild	1.5	(1.2–1.8)	6.1	(4–7.1)	3.0	0	11	(3.1–22)	11	(6.0–15)	9.0	(4.9–15)	0	
M19T	20/20	Very mild	0.8	(0.1–1.4)	10	(9.0–12)	<1.0	0	11	(9.8–12)	19	(9.0–26)	24	(3.2–46)	25	(19–31)
M1te;M19T	10/10	Very mild	0.4	(0.4–0.5)	3.5	(3.4–3.5)	<1.0	0	15	(0.8–35)	15	(4.6–25)	33	(7.0–44)	0	
In21S	20/20	Very mild	0.4	(0.01–1)	7.8	(0.2–13)	<1.0	0	20	(5.2–39)	30	(11–72)	16	(2.2–32)	0	
Y24te	16/16	Very mild	0.8	(0.05–1.8)	5.6	(3.9–7.3)	<1.0	0	11	(6.8–18)	19	(14–27)	46	(30–67)	0	
C84S;C86S	19/19	Mild	18	(12–26)	46	(27–102)	51	0	25	(3.6–62)	41	(17–95)	23	(5.7–53)	4.3	(0–8.0)
Q104te	10/10	Severe[e]	41	(23–67)	59	(40–83)	101	90	26	(8.1–46)	17	(6.9–30)	24	(9.3–44)	17	(7.6–26)
Δ121-137	20/20	Very mild	0.8	(0.3–1.4)	6.3	(4.8–7.3)	<1.0	0	139	(66–228)	158	(102–242)	93	(79–118)	0	
Δ121-137;M19T	9/9	Very mild	0.8	(0.6–0.9)	4.2	(3.5–5.2)	<1.0	0	14	(13–16)	13	(11–15)	26	(18–45)	0	
Δ121-137;Y24te	Not tested	—	—		—		—	—	10	(2.3–18)	7.0	(2.0–12)	19	(14–23)	0	
Δ121-184	10/10	Very mild	1.0	(0.1–1.6)	4.7	(0.8–8.7)	3.0	0	48	(22–98)	58	(34–110)	44	(9.8–73)	0	
Δ127-286	10/10	Very mild	0.6	(0.1–1.4)	4.6	(3.5–6.7)	<1.0	0	61	(50–84)	82	(77–89)	74	(30–120)	0	
Inv121-131	Not tested	—	—		—		—	—	24	(9.6–52)	29	(17–52)	35	(18–65)	0	
Δ121-126	10/10	Mild	47	(24–76)	53	(39–79)	40	1.6	82	(73–90)	150	(143–157)	141	(113–169)	0	
CP mutants																
R66fr	10/10	Severe[f]	2.9	(0–4.8)	75	(48–112)	0	53	6.6	(1.7–18)	326	(272–365)	0		94	(89–99)
C68S;C72S	10/10	Severe	10	(6.1–15)	82	(81–109)	23	35	5.1	(2.6–7.5)	479	(437–521)	10	(0–15)	89	(76–102)
H85K	5/5	Severe	53	(39–70)	95	(84–112)	98	74	54	(19–88)	145	(103–187)	51	(19–74)	91	(84–97)
T251te	5/5	Severe	1.6	(0.4–3.5)	71	(33–122)	<1.0	6.9	17	(2.0–35)	700	(481–840)	28	(4.5–66)	103	(66–141)
AV2/CP double mutants																
M1te/R66fr	10/10	Very mild	0.3	(0.05–0.5)	11	(9.6–17)	0	0	0.02	(0–0.03)	506	(427–584)	0		0	
M19T/R66fr	10/10	Very mild	0.04	(0.01–0.07)	11	(9.4–13)	0	0	0.042	(0–0.04)	444	(437–521)	0		0	
Δ121-137/R66fr	10/10	Very mild	0.02	(0–0.2)	14	(3.7–35)	0	0	0.6	(0–1.2)	112	(95–128)	0		0	
Y61te/S11te	15/15	Very mild	0.05	(0–0.2)	10	(2.7–17)	0	0	4.5	(2.2–6.6)	283	(254–303)	13	(0–21)	0	
ΔAV2CP-GFP	10/10	Very mild	0.04	(0–0.1)	4.4	(3.1–7.6)	0	0	7.5	(0–15)	566	(363–769)	0		0	
Other mutants																
AC1M	0/10	No symptoms	0		0		0	0	0		0		0		0	
AC2M	10/10	Very mild	1.0	(0.4–2.1)	0.9	(0.4–1.4)	5.0	0	13	(8.6–18)	273	(259–288)	5.4	(3.4–8.0)	0	
AC3M	10/10	Mild	31	(19–49)	21	(7.8–43)	81	6.5	1.3	(1.1–2.1)	3.1	(1.6–5.4)	0		0	
AC23M	10/10	Very mild	1.0	(0.2–1.9)	1.0	(0.2–1.6)	<1.0	0	0.5	(0.2–0.8)	0.3	(0–0.5))	0		0	
BV1M	10/10	No symptoms	0.4		0.3		<1.0	0	Not tested		—		—		—	
BC1M	0/10	No symptoms	0		0		0	0	Not tested		—		—		—	
	Tomato															
Wild type	10/10	Severe	100	(72–119)	100	(65–132)	100	100								
AV3-M1R	10/10	Severe	56	(47–66)	86	(71–99)	82	Not tested								
AV2-Y24te	10/10	Very mild	0.6	(0.2–0.9)	11	(0.5–17)	<1.0	Not tested								
AV2-Δ121-137	10/10	Very mild	0.2	(0.1–0.3)	8.0	(1.5–14)	<1.0	Not tested								
CP-R66fr	10/10	Severe	1.3	(1.1–1.5)	84	(76–90)	0	Not tested								
Y61te/S11te	10/10	Very mild	0.8	(0.6–0.9)	16	(2.5–47)	0	Not tested								

[a] The amounts of viral DNA and CP and AV2 protein in plants and protoplasts inoculated with the wild-type viral DNA were assigned a value of 100.

[b] The values represent the average amount (range) of single-stranded (ss) and double-stranded (ds) A-component DNA in 4 inoculated plants per mutant, except for AV3 mutants BV1M and BC1M. Values for AV3 mutants BV1M and BC1M represent the amount of viral DNA in 10 plants per mutant pooled together. Viral DNA was quantitated using the 'phosphorimager' from Molecular Dynamics.

[c] The values represent the amount of CP and AV2 detected in inoculated plants. Protein extracts were prepared from the same plants that were used for DNA isolation. CP and AV2 were detected on Western blots with ECL detection method and the signal on X-ray film was quantitated using the NIH Image program.

[d] The values are average (range) amounts of viral DNA and CP and AV2 protein detected in three to four independent protoplast transfections per mutant, except for AV/CP double mutants for which the values are average of two transfections.

[e] Severe symptoms without drastic reduction in internodal length.

[f] Severe symptoms without intense chlorosis.

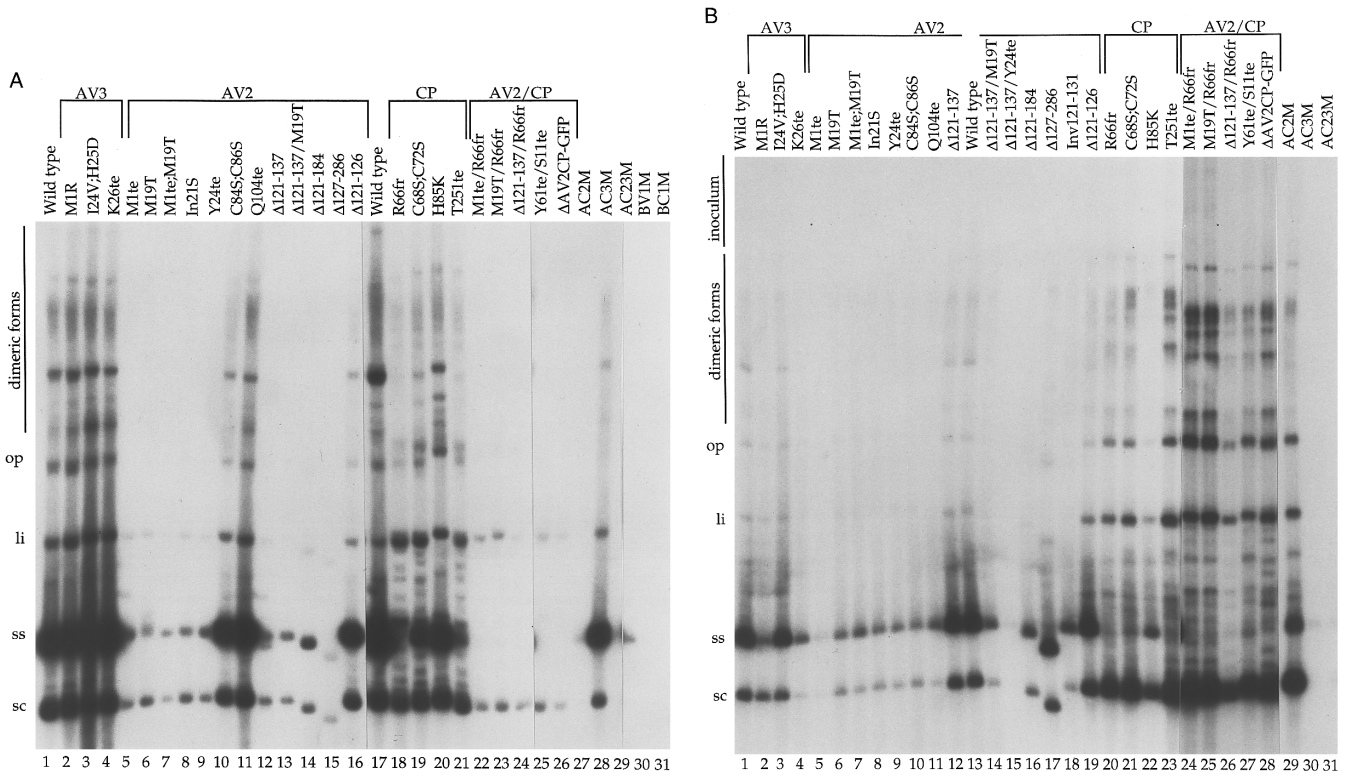

FIG. 2. Southern blot analysis of viral DNA in inoculated plants and protoplasts. DNA was electrophoresed through 1% agarose gel (without ethidium bromide), transferred to nylon membrane, and hybridized with ^{32}P-labeled A-component-specific probe. The positions of single-stranded (ss), supercoiled (sc), open (op), and linear (li) viral DNA forms are indicated. (A) Each lane contains 1 μg of DNA prepared from systemically infected leaves of a single plant, except in lanes 1–5, 30, and 31 which have 1 μg DNA prepared from a pool of 10 plants. (B) Each lane contains 2 μg DNA prepared from protoplasts (single transfection).

Since mutations in AV3 did not affect viral DNA accumulation and symptom development in plants we used 5′ RACE PCR to determine whether or not a transcript could be identified that indicated that ORF AV3 is transcribed. Of the 66 5′ RACE PCR clones analyzed for insert length on agarose gels, 33 had ~300-bp inserts, 17 had ~200-bp inserts, and 16 had ~80-bp inserts. The ~300-bp inserts were less than the expected length (~380 bp) of transcripts initiating at the ATG site of ORF AV3. Five clones with ~300-bp inserts, 3 clones with ~200-bp inserts, and 3 clones with ~80-bp inserts were sequenced and the initiation sites are shown in Fig. 1B. None of the transcripts initiated 5′ of the ATG of ORF AV3. It was therefore concluded that ORF AV3 does not encode a protein.

Mutations in AV2 attenuate symptoms, and plants accumulate reduced levels of viral DNA

Among the mutations introduced into AV2 which encode a putative protein of 112 aa (Table 1), mutation M1te;M19T prevented the translation of the AV2 protein. Mutation M1te prevents initiation at the first initiation codon while M19T eliminates a second potential initiation codon 54 nt downstream of the first initiation codon (Fig. 1B). Mutations Y24te and Q104te prematurely terminate the AV2 protein after aa 23 and aa 103, respectively. Cys84 and Cys86 were converted to Ser residues in mutation C84S;C86S with the intent to affect the dimerization of the protein. (Results presented later show that AV2 exists as monomer, dimer, and multimer forms in inoculated plants.) In mutation In21S a Ser residue was introduced following Leu20. The deletion mutant Δ121-137 was constructed by deleting a 17-bp *Bam*HI fragment. This resulted in the deletion of nt −6 to +11 of the ORF AV2 (Fig. 3).

In general, *N. benthamiana* plants inoculated with virus DNA that contained mutations in AV2 developed very mild symptoms, with the exception of plants inoculated with mutants C84S;C86S and Q104te (Table 2). Symptoms included very mild chlorosis and leaf curling without reductions in leaf size and internodal lengths. Symptoms were so mild that often it was difficult to distinguish inoculated from mock-inoculated plants. Plants inoculated with the mutant C84S;C86S showed mild symptoms with moderate chlorosis and some reduction in leaf size and internodal length; while mutant Q104te was as infectious as the wild-type virus and induced severe symptoms but with less pronounced reduction in internodal length. To-

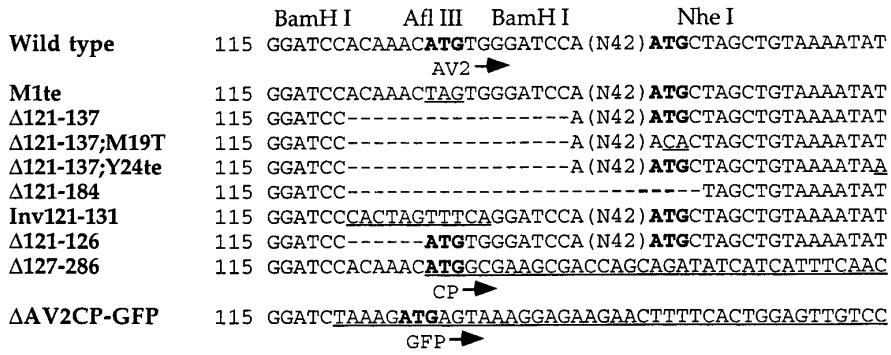

FIG. 3. Mutations in ORFs AV2 that modified sequence around the initiation codon of AV2. Nucleotide changes from the wild-type sequence are underlined. Potential initiation codons are shown in bold. Restriction sites are shown above the wild-type sequence.

mato plants inoculated with mutants Y24te and Δ121-137 also showed very mild symptoms (Table 2).

Plants inoculated with mutants M1te, M19T, M1te;M19T, In21S, Y24te, and Δ121-137 accumulated reduced levels of viral DNA (Fig. 2A, lanes 5–9 and 12). The amount of ssDNA ranged from 0.01 to 1.8% of the amount in wt infections while dsDNA ranged from 0.2 to 13% of the amount in plants inoculated with wt DNA (Table 2). Plants inoculated with mutant C84S;C86S accumulated, on average, 18% of ssDNA and 46% of dsDNA of those produced by wt virus (Fig. 2A, lane 10; Table 2). Plants inoculated with mutant Q104te accumulated ss and dsDNA to 41 and 59% of wt levels, respectively (Fig. 2A, lane 11; Table 2). Similar results were obtained when DNA prepared from individual plants and from the pools of the remaining plants from each of the mutants was analyzed (data not shown).

Mutations in CP do not affect symptom development but reduce ssDNA accumulation in plants

Mutation R66fr is identical to the CPM1 mutation reported earlier (Padidam *et al.,* 1995a) and truncates the CP after aa 65. Mutations C68S;C72S and H85K were made within the potential zinc finger sequence that is conserved in the CPs of WTGs (Fig. 4). Mutation T251te, created by the introduction of a *Hin*dIII site, terminates the CP 6 aa prematurely.

All the mutants induced severe symptoms when inoculated onto plants (Table 2). The development and severity of symptoms were similar to those in plants inoculated with wt DNA. Plants infected with R66fr, C68S:C72S, T251te, and H85K accumulated dsDNA to wt levels but with markedly diminished accumulation of ssDNA (Fig. 2A, lanes 18–21). The accumulation of ssDNA in plants infected with R66fr, C68S:C72S, and T251te ranged from 0 to 15% of the wild-type level while in those infected with mutant H85K ssDNA accumulation was intermediate ranging from 39 to 70% of the wt level (Table 2). This suggests that mutation H85K does not completely abolish the function of the CP in accumulation of ssDNA. Mutant R66fr elicited similar response in both *N. benthamiana* and tomato plants (Table 2).

Mutations in both AV2 and CP attenuate symptoms, and plants accumulate reduced levels of viral DNA

Five different AV2/CP double mutants were constructed (Table 1). Mutants M1te/R66fr, M19T/R66fr, and Δ121-137/R66fr have the potential to code for a shorter AV2 (initiating at Met19 in M1te/R66fr and Δ116-132/R66fr) or mutated AV2 (in M19T/R66fr) and a CP that is truncated at aa 69. In Y61te/S11te mutant, AV2 and CP are terminated after aa 61 and 11, respectively. CP synthesis, however, can potentially initiate at Met49 in this mutant. In mutant ΔAV2CP-GFP the entire coding sequence of ORF AV2 and CP was replaced by a sequence coding for the green fluorescent protein (GFP; Chalfie *et al.,* 1994), beginning 7 bp before the ORF AV2.

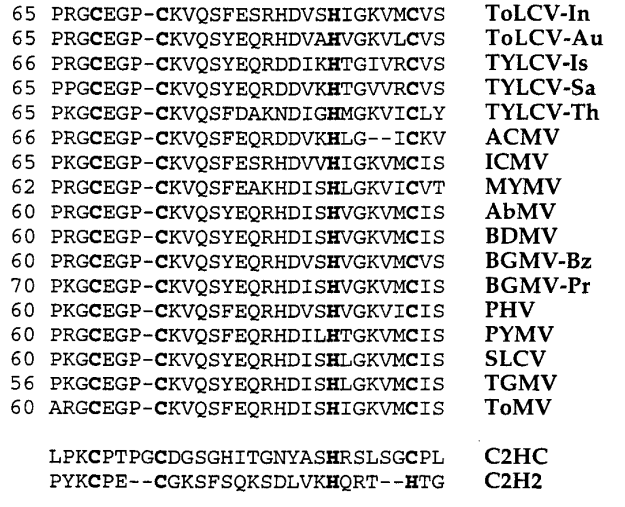

FIG. 4. Alignment of putative zinc finger domains of coat proteins of whitefly-transmitted geminiviruses. Consensus sequences of C2HC type (from yeast transcription factor Myt1; Kim and Hudson, 1992) and C2H2 type (based on a database of 131 zinc finger sequences; Krizek *et al.,* 1991) zinc fingers are also shown. Proposed ligands are shown in bold. Geminivirus sequence references and GenBank Accession No. are given in Padidam *et al.* (1995b).

The symptoms elicited by the five double mutants were essentially identical to the very mild symptoms elicited by AV2 mutants (Table 2). Inoculated plants accumulated low levels of both ss and dsDNA as with AV2 mutants (Fig. 2A, lanes 22–26; Table 2). The reduction in ssDNA was, however, much greater than in plants inoculated with either AV2 or CP mutants alone and many inoculated plants lacked ssDNA.

Deletion of sequences around the Met1 codon of AV2 do not affect viral DNA replication in protoplasts

Low levels of viral DNA accumulation in plants inoculated with virus that contain mutations in AV2 could be due to impaired DNA replication, or viral movement, or both. To determine the effects on virus replication, each mutant virus was introduced into protoplasts derived from BY-2 cells. Mutations M1te, M19T, M1te;M19T, In21S, Y24te, C84S;C86S, and Q104te in AV2 affected the replication of both ss and dsDNA in protoplasts (Fig. 2B, lanes 5–11). On average, these mutations caused 2- to 10-fold reductions in DNA accumulation (Table 2). Surprisingly, protoplasts inoculated with the AV2 mutant Δ121-137 (deletion of nt −6 to +11 of ORF AV2) accumulated both ss and dsDNA to levels slightly higher than those in protoplasts inoculated with wt DNA (Fig. 2B, lane 12; Table 2) even though inoculated plants had shown very mild symptoms and greatly reduced amounts of viral DNA. Additional mutations were made in this region to further study this observation (Fig. 3). In mutation Δ121-126, the 6 nt immediately before the first ATG of ORF AV2 were deleted; in mutation Δ121-184 the deletion extended from −6 to +58 nt of ORF AV2 to include the second potential initiation codon. In mutation Δ127-286, the sequence following the first ATG of ORF AV2 to the ATG of CP was deleted, thus placing the ATG of the CP ORF at the same location as the ATG of ORF AV2. In mutation Inv121-131, the 17-bp *Bam*HI fragment of the mutant M1te was inverted, thus changing 6 nt upstream of the ATG of ORF AV2 and resulting in replacement of codons for Met1 and Tyr2 with Phe and Val. Mutations Δ121-137;M19T and Δ121-137;Y24te were constructed to prevent the initiation of AV2 from Met19, and to terminate the protein at Tyr24, respectively. Among the above mutants only mutant Δ121-126 has the entire coding region of AV2 unmodified.

Inoculation of *N. benthamiana* plants with mutants Δ121-137;M19T, Δ121-184, and Δ127-286 resulted in very mild symptoms and accumulation of low titers of ss and dsDNA. These results were similar to those from plants inoculated with mutants M1te and Δ121-137 (Fig. 2A, lanes 13–15; Table 2). Interestingly, protoplasts inoculated with the mutants Δ121-137;M19T and Δ121-137;Y24te accumulated low levels of viral DNA (7–14% of the wt levels) unlike the protoplasts inoculated with the mutant Δ121-137 which accumulated viral DNA to wt levels (Fig. 2B, lanes 12–15; Table 2). Protoplasts inoculated with mutants Δ121-184 and Δ127-286 (which also do not have the potential to encode AV2) accumulated viral DNA to ~50 and 75% (on average) of the wt level, respectively (Fig. 2B, lanes 16 and 17; Table 2). Protoplasts inoculated with the mutant Inv121-131 accumulated viral DNA on average to ~25% of the wt level (Fig. 2B, lane 18; Table 2). Plants inoculated with the mutant Δ121-126 developed mild symptoms and accumulated viral DNA to approximately 50% of wt levels (Fig. 2A, lane 16; Table 2), while protoplasts inoculated with this mutant accumulated viral DNA to wt levels (Fig. 2B, lane 19; Table 2).

Mutations in CP increase the accumulation of dsDNA in protoplasts and are dominant over mutations in AV2

Protoplasts were inoculated with CP mutants and AV2/CP double mutants to determine if protoplasts also accumulate low levels of ssDNA as in plants inoculated with the CP and AV2/CP mutants. The accumulation of ssDNA in protoplasts inoculated with the CP mutants R66fr, C68;C72S, H85K, and T251te was comparable to the levels observed in plants inoculated with those mutants (Fig. 2B, lanes 20–23; Table 2). However, the level of dsDNA accumulation in the mutants, in general, was three- to eightfold higher than in protoplasts inoculated with wt DNA, with the exception of the mutant H85K. Interestingly, protoplasts inoculated with the AV2/CP double mutants also accumulated high levels of dsDNA (Fig. 2B, lanes 24–28; Table 2). These results show that the absence of wt CP, irrespective of the presence or absence of AV2, can result in marked increase in the replication of dsDNA. Protoplasts inoculated with the AV2/CP double mutant Δ121-137/R66fr did not accumulate high levels of dsDNA (Table 2).

AV2 protein does not complement AV2 mutants *in trans*

The low levels of viral DNA replication in protoplasts transfected with AV2 mutants M1te, M19T, M1te;M19T, In21S, Y24te, C84S;C86S, and Q104te suggests that AV2 protein promoted efficient replication. However, AV2 deletion mutants showed wild-type or near wild-type levels of replication (Fig. 2B). To resolve this apparent contradiction, protoplasts were cotransfected with AV2 mutants M1te, M19T, M1te;M19T, Δ121-137, or Δ121-137;M19T and plasmids containing AV2, CP, or AV2 and CP gene expression cassettes (i.e., coding sequences cloned between a duplicated 35S promoter and NOS polyadenylation sequences; described in Table 1). As shown in Table 3, cotransfection with a plasmid containing the AV2 expression cassette (35S-AV2) had no effect on the replica-

TABLE 3

Summary of Results Obtained with the Complementation Experiments in *N. tabacum* Protoplasts[a]

Coinoculum[b]	AV2-M1te		AV2-M19T		AV2-M1te;M19T		AV2-Δ121-137		AV2-Δ121-137;M19T		CP-R66fr	
	ssDNA	dsDNA	ssDNA	dsDNA	ssDNA	dsDNA	ssDNA	dsDNA	ssDNA	dsDNA	ssDNA	dsDNA
None	13 (6.7–30)	19 (12–35)	13 (5.1–26)	32 (17–41)	13 (3.1–23)	18 (7–25)	101 (62–208)	148 (104–199)	12 (7.8–17)	22 (9.5–41)	3.6 (0.6–11)	463 (287–636)
35S-AV2	3.5 (2.5–4.4)	10 (7.5–13)	18 (17–19)	30 (29–31)	12 (4.6–24)	30 (17–38)	116 (60–173)	204 (165–244)	11 (6.0–19)	14 (9.8–16)	5.5 (2.7–11)	351 (110–740)
35S-CP	0	27 (22–32)	0.3 (0–0.5)	26 (22–30)	0.5 (0–0.9)	22 (7.8–39)	1.4 (0.2.8)	71 (63–71)	1.4 (1.1–1.6)	50 (48–51)	1.1 (1.0–1.1)	474 (462–486)
35S-AV2CP	0.5 (0–0.9)	27 (9.0–45)	0.2 (0.1–0.2)	22 (19–24)	0.3 (0–0.5)	56 (55–56)	20 (14–26)	216 (202–229)	1.2 (0.4–1.9)	23 (13–32)	1.0 (0.6–1.3)	389 (343–430)
35S-AV2$^-$CP$^-$	2.0 (0–5.9)	9.5 (8–11)	0.1 (0–0.2)	15 (11–21)	0.5 (0–1.0)	26 (5.0–50)	7.0 (1.1–13)	231 (142–320)	3.9 (0.5–8.9)	23 (11–31)	2.5 (1.8–3.2)	328 (270–385)
AC1M[c]	0.1 (0–0.2)	8.3 (7–9.4)[d]	0.1 (0–0.2)	20 (14–25)[d]	0.7 (0–1.4)	29 (26–32)[d]	27 (12–39)[e]	139 (90–168)[e]	0.4 (0.2–0.5)	12 (11–12)[d]	30 (19–40)[f]	176 (168–184)[f]

[a] The values are average (range) amounts of single-stranded (ss) and double-stranded (ds) A-component DNA detected in four independent experiments for transfections without coinoculations, three for transfections with 35S-AV2 and 35S-AV2$^-$CP$^-$ coinoculations, and two for transfections with other coinoculations. The amount of viral DNA in protoplasts inoculated with the wild-type viral DNA was assigned a value of 100. Viral DNA was quantitated using the phosphorimager from Molecular Dynamics.

[b] forty μg of 35S construct DNA or 10 μg of AC1M DNA was coinoculated with 2 μg of AV2 or CP mutant DNA.

[c] AC1M mutant viral DNA is 60 bp shorter than AV2 and CP mutant DNAs and migrates ahead of AV2 and CP mutant viral DNAs in 1% agarose gels.

[d] All the dsDNA detected was that of AC1M.

[e] Thirty percent of ssDNA and 60% of dsDNA detected was that of AC1M.

[f] Forty percent of ss and dsDNA detected was that of AC1M.

tion of AV2 mutants. Surprisingly, cotransfection with a plasmid containing CP (35S-CP) or AV2 and CP (35S-AV2CP) expression cassettes blocked the accumulation of ssDNA without affecting the accumulation of dsDNA of AV2 mutants. Similar results were also obtained when protoplasts were cotransfected with AV2 mutants and a plasmid containing an expression cassette that produced AV2 and CP mRNA but not the respective proteins (35S-AV2$^-$CP$^-$). These results suggested that A-component virion-sense mRNA can inhibit ssDNA accumulation.

We also cotransfected protoplasts with CP mutant R66fr and the plasmids that contain the gene expression cassettes. As expected, cotransfection with the 35S-AV2 plasmid had no effect on replication of CP mutant DNA (Table 3). Cotransfection of protoplasts with the CP mutant R66fr and a plasmid containing a 35S-CP or 35S-AV2CP expression cassette did not increase the accumulation of ssDNA of this mutant DNA (Table 3). This result is expected if the genes 35S-CP and 35S-AV2CP inhibit ssDNA accumulation, as observed with AV2 mutants.

The lack of complementation by the 35S-AV2 gene expression plasmid of the AV2 mutants, and between the 35S-CP plasmid and CP mutant, could be due to insufficient AV2 and CP synthesis from these chimeric genes. To address this question, we cotransfected protoplasts with the AV2 mutant M1te;M19T and CP mutant R66fr. In these experiments protoplasts accumulated reduced levels of ssDNA as expected for AV2 mutant M1te;M19T and increased levels of dsDNA as expected for CP mutant R66fr (data not shown). In addition, we cotransfected protoplasts with AV2 or CP mutants and the AC1 mutant (AC1M). AC1M mutant DNA will replicate only in the presence of AV2 or CP mutant DNA since the AC1 protein is essential for replication (see below). Surprisingly, coinoculation of protoplasts with AV2 mutants M1te, M19T, M1te;M19T, or Δ121-137;M19T and AC1M resulted in the accumulation of low levels of only AC1M mutant dsDNA (Table 3). In contrast, protoplasts cotransfected with the AV2 mutant Δ121-137 or CP mutant R66fr and AC1M accumulated ss and dsDNA viral DNAs of both mutants (Table 3). These results showed that CP or AC1 mutants failed to complement AV2 mutants.

AV2 protein exists as monomer, dimer, and multimer in plants

Plants inoculated with wt viral DNA expressed AV2 protein in readily detectable amounts on a Western blot (Fig. 5A, lane 1). In addition to the expected 13-kDa band, bands corresponding to dimer, trimer, and other multimer forms were detected. The sample preparation buffer contained 2% SDS and 5% β-mercaptoethanol and samples were boiled for 6 min before loading. The multimeric

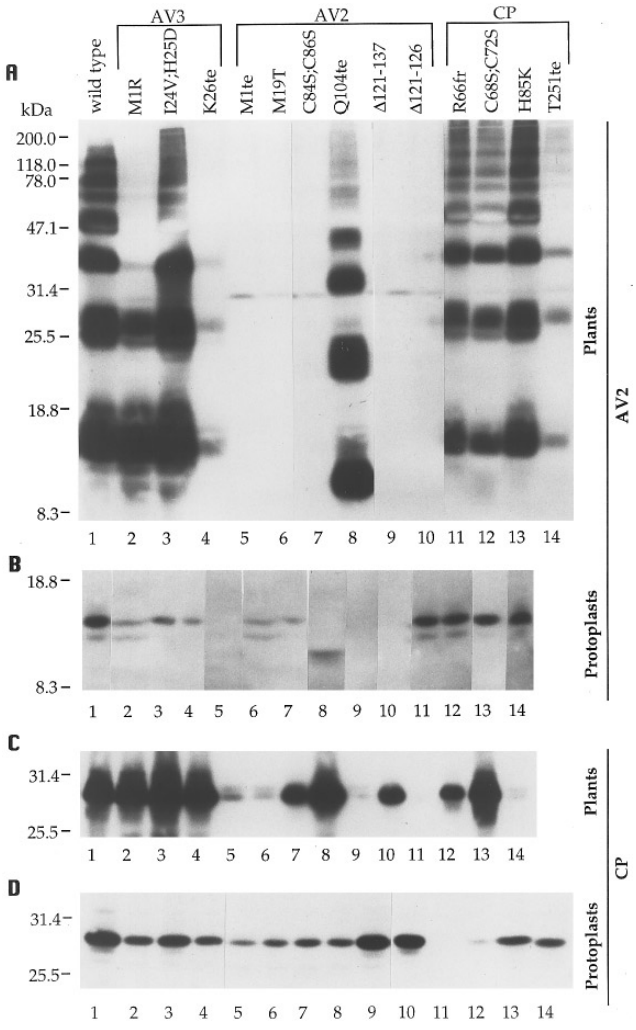

FIG. 5. Western blot detection of AV2 and coat protein in inoculated plants and protoplasts. Protein extracts prepared from systemically infected leaves or infected protoplasts were separated on SDS–PAGE and transferred to Immobilon-P membrane (Millipore), and AV2 or CP was detected using the anti-AV2 or anti-CP antisera with the ECL detection method. (A) Detection of AV2 in plants. Each lane contained 20 μg of protein prepared from pooled leaves of 4 to 10 plants per mutant. (B) Detection of AV2 in protoplasts. Each lane contained 100 μg of protein from single transfection. (C) Detection of CP in plants. Each lane contained 5 μg of protein prepared from pooled leaves of 4 to 10 plants per mutant. (D) Detection of CP in protoplasts. Each lane contained 50 μg of protein from single transfection.

forms were also detected when samples were loaded without boiling. In contrast, dimeric and multimeric forms were not detected in protoplasts inoculated with the wt viral DNA (data not shown).

Plants and protoplasts inoculated with the AV3, AV2, CP, and AV/CP double mutants were analyzed by Western blotting to correlate symptom development and viral DNA accumulation to levels of AV2 protein. The plants and protoplasts inoculated with the AV3 mutants, with the exception of protoplasts inoculated with mutant K26te, accumulated AV2 protein to levels similar to those resulting from infection of wt virus (Figs. 5A and 5B; Table 2). Plants inoculated with AV3 mutant K26te accumulated AV2 protein to only 10% of the level found in plants inoculated with the wt DNA. Among the plants inoculated with the AV2 mutants, AV2 was only detected in plants inoculated with the mutants Q104te and Δ121-126 (Fig. 5A; Table 2). Plants inoculated with the mutant Q104te expressed the expected truncated protein of 10.8 kDa. AV2 was detected only in protoplasts inoculated with the AV2 mutants M19T, C84S;C86S, and Q104te (Fig. 5B and Table 2). AV2 was also detected in plants and protoplasts inoculated with the CP mutants at levels that varied from 6.9 to 103% of the wt level (Figs. 5A and 5B; Table 2). No AV2 protein was detected in plants and protoplasts inoculated with the AV2/CP double mutants. Up to 500 μg protein from protoplasts and 300 μg of protein from plants inoculated with AV2 mutants was analyzed on Western blots in attempts to detect AV2 protein.

Mutations in AV2 and AV3 do not prevent CP synthesis

Anti-CP rabbit antibodies detected a protein of an estimated size of 29 kDa in plants and protoplasts inoculated with viral DNA that contained mutations in AV2 or AV3 (Figs. 5C and 5D). In general, the amount of CP detected on Western blots correlated with the amount of viral dsDNA detected in inoculated plants and protoplasts (Table 2).

Plants and protoplasts inoculated with viral DNA with mutations only in the CP, or in both AV2 plus CP, were also assayed for CP accumulation. No CP was detected in plants and protoplasts inoculated with CP mutant R66fr (Figs. 5C and 5D, lane 11) and AV2/CP double mutants M1te/R66fr, M19T/R66fr, and ΔAV2CP-GFP (Table 2). A protein of ~24 kDa, the size of protein predicted for a CP initiated at Met49, was detected in protoplasts inoculated with the AV2/CP double mutant Y61te/S11te (Table 2). CP was readily detected in plants and protoplasts inoculated with the mutants C68S;C72S, H85K, and T251te (Figs. 5C and 5D, lanes 12–14; Table 2).

AC1, AC2, AC3, BV1, and BC1 are essential for ToLCV-India infection

An ORF corresponding to the AV2 of ToLCV-India is absent in WTGs from the New World. This raised the question about the roles of other ORFs of ToLCV-India in comparison with the corresponding ORFs in other WTGs. Mutation in ORF AC1 (AC1M) abolished viral replication (Table 2). Plants inoculated with the AC2 mutant (AC2M) developed very mild symptoms (Table 2) and accumulated low levels of ss and dsDNA even though the inoculated protoplasts accumulated dsDNA to threefold higher levels than infection with wt virus (Fig. 2A, lane 27; Fig. 2B, lane 29). Only 5.4% (on average) of wt levels of CP

was detected in protoplasts inoculated with the mutant AC2M despite the fact that the protoplasts accumulated dsDNA to 270% (on average) of wt levels (Table 2). Thus, the AC2 product appears to be required for the transactivation of AV2 and CP genes of ToLCV-India. Plants inoculated with the AC3 mutant (AC3M) developed mild symptoms and accumulated on average ssDNA to 31% and dsDNA to 21% of wt levels (Table 2). Protoplasts, in contrast, accumulated ss and dsDNA to only 1.3 and 3.1% (on average) of levels caused by wt virus, respectively (Fig. 2A, lane 28; Fig. 2B, lane 30). The virus in which both AC2 and AC3 were mutated (AC23M) behaved like mutant AC2M in plants and like mutant AC3M in protoplasts (Fig. 2A, lane 29; Fig. 2B, lane 31). Mutations in BV1 (BV1M) and BC1 (BC1M) abolished symptom development in plants; plants inoculated with BV1M but not BC1M accumulated very low levels of viral DNA (Fig. 2A, lanes 30 and 31; Table 2).

DISCUSSION

We analyzed various mutants of ToLCV-India to ascertain the role of AV3, AV2, and CP in viral replication, movement, and symptom development. Several lines of evidence indicate that the putative ORF AV3 probably does not encode a protein that is important for any of these processes. First, we did not identify a transcript whose 5′ end encompasses the putative initiation codon of the ORF. Second, both *N. benthamiana* and tomato plants inoculated with AV3 mutants developed severe symptoms and accumulated viral DNA to levels similar to those of plants inoculated with the wt viral DNA. Third, results obtained with AV2 mutants are consistent with the hypothesis that ORF AV3 does not encode a protein. For example, AV2 mutant Y24te did not alter the aa sequence of AV3, and elicited responses in plants and protoplasts that were similar to those with the AV2 mutant M1te in which predicted His27 of AV3 was mutated to Leu. Nevertheless, there were low levels of ssDNA in protoplasts inoculated with the AV3 mutant M1R and low levels of ss and dsDNA in protoplasts inoculated with the AV3 mutant K26te. The first may be explained by an effect of M1R on viral replication: this mutation resulted in nucleotide change in the sequence near the conserved stem–loop that is required for geminivirus replication (Fontes *et al.,* 1994). The AV3 mutant K26te may affect the levels of AV2 mRNA transcription or translation, since plants and protoplasts inoculated with mutant K26te accumulated low levels of AV2 protein (Fig. 5).

Among the more than 40 geminivirus isolates sequenced to date, an ORF in a position similar to that of AV3 in ToLCV-India is found only in beet curly top virus (BCTV; ORF R3) and was shown to be required for efficient viral movement (Frischmuth *et al.,* 1993; Hormuzdi and Bisaro, 1993). There is no significant homology between AV3 and R3 of BCTV or any other sequence available in the database. We conclude that the ORF AV3 of ToLCV-India either exists fortuitously or may have lost its function during viral evolution.

Mutations in CP caused a marked decrease in ssDNA accumulation in *N. benthamiana* and tomato plants and in *N. tabacum* protoplasts while increasing dsDNA accumulation in protoplasts. In similar studies low levels of ssDNA, but not increased levels of dsDNA, were observed in plants inoculated with CP mutants of African cassava mosaic virus (ACMV; Stanley and Townsend, 1986; Etessami *et al.,* 1989), maize streak virus (MSV; Boulton *et al.,* 1989; Lazarowitz *et al.,* 1989), and ToLCV-Australia (Rigden *et al.,* 1993), and in protoplasts inoculated with CP mutants of BCTV (Briddon *et al.,* 1989), MSV (Lazarowitz *et al.,* 1989), and wheat dwarf virus (Woolston *et al.,* 1989). On the other hand, TGMV CP mutations had no effect on DNA accumulation in plants (Brough *et al.,* 1988; Gardiner *et al.,* 1988), but reduced ssDNA accumulation while slightly increasing the dsDNA accumulation in protoplasts (Sunter *et al.,* 1990). CP may regulate the accumulation of ssDNA such that absence of CP early in infection results in continuous dsDNA synthesis and its presence later in the infection cycle results in virus encapsidation. Hence, lack of CP would result in increased dsDNA synthesis by increasing the amount of template available for minus-strand synthesis. Our results show such an increase in dsDNA due to mutations in CP and provide support for a role of CP in DNA replication.

It is possible that the interaction of the CP with DNA involves the putative zinc finger sequences conserved among the CPs of the WTGs. Many proteins with zinc finger domains have been studied extensively and shown to have specific nucleic acid binding functions; some of these proteins are involved in aspects of eukaryotic gene regulation (Schmiedeskamp and Kelvit, 1994). Mutations in the putative zinc finger sequence of CP of ToLCV-India affected DNA accumulation but to varying degrees. While mutating the Cys68 and Cys72 to Ser disrupted the function of CP and altered the accumulation of normal levels of ss and dsDNA, mutating His85 to Lys only partially disrupted the accumulation of DNA. Furthermore, CP with His85Lys mutation accumulated in inoculated plants to levels comparable to CP in plants infected with wt virus. Further experiments are needed to establish the specific role of putative zinc finger sequence in CP:DNA interactions and its impact on accumulation of ss and dsDNA.

Mutations in ORF AV2 clearly demonstrated its requirement for the accumulation of viral DNA and in symptom development in plants. Plants inoculated with AV2 mutants and AV2/CP double mutants accumulated low levels of viral DNA and showed very mild or no symptoms. Although the role of AV2 in symptom development is difficult to assess on the basis of our results,

the severity of symptoms in plants was positively correlated with the levels of viral DNA accumulation. The very mild symptoms in plants could be an indirect effect of low levels of viral DNA, or low levels of other viral factors, including AV2.

The low levels of viral DNA accumulation in plants could result from impaired viral movement or replication. In general, low levels or no accumulation of mutant virus in plants coupled with normal accumulation in protoplasts suggests a role for the protein in viral movement; similarly, accumulation of low levels of mutant DNA both in plants and in protoplasts suggests a role for the protein in viral replication. By these criteria virus with deletion mutations in AV2 ORF clearly demonstrated that AV2 protein is required for efficient viral movement. However, the results obtained for AV2 mutants M1te, M19T, M1te;M19T, In21S, Y24te, C84S;C86S, and Q104te are not in agreement with those observed for the AV2 deletion mutants. The latter group of mutants accumulated to low levels in both protoplasts and plants, suggesting a role for AV2 protein in viral replication. However, the following observations argue against a role for AV2 protein in viral replication. First, AV2 mutants were not complemented by A component that included CP mutant R66fr or a plasmid containing an AV2 expression cassette. Second, preventing CP expression (e.g., AV2/CP mutants M1te/R66fr and M19T/R66fr) increased the replication (i.e., the levels of dsDNA) of AV2 mutants M1te and M19T up to three- to sixfold higher than the wt level. If AV2 is required for viral replication, mutation in CP should not have increased the viral replication.

AV2 is well conserved in WTGs from the Old World and the percentage aa identity between ToLCV-India and other viruses ranges from 45 to 66% (Padidam *et al.,* 1995a). Disruption of AV2 in ToLCV-Australia (which has a monopartite genome) led to symptomless, systemic infection with reduced titer of all DNA forms (Rigden *et al.,* 1993), but disruption of the AV2 ORF in ACMV (which has a bipartite genome) had no effect on infection (Etessami *et al.,* 1989). Mutations in an ORF present in a similar location in leafhopper-transmitted geminiviruses showed that this ORF is required for cell to cell spread by MSV (Boulton *et al.,* 1989; Lazarowitz *et al.,* 1989) and not required for spread by BCTV (Hormuzdi and Bisaro, 1993; Stanley *et al.,* 1992). It is difficult to draw any comparison between AV2 of ToLCV-India and the ORF present in MSV and BCTV because of lack of sequence similarity between these proteins.

In WTGs with bipartite genomes, BV1 and BC1 are known to promote viral movement and symptom development (Brough *et al.,* 1988; Etessami *et al.,* 1988; Ingham *et al.,* 1995). Mutations in BV1 and BC1 of ToLCV-India abolished symptom development. The requirement of AV2, BV1, and BC1 for spread and symptom development shows that multiple essential functions are needed for ToLCV-India movement.

The role of AC1, AC2, and AC3 in the infectious cycle of ToLCV-India appears to be similar to the corresponding ORFs present in other WTGs. Genetic analysis of Abutilon mosaic virus (Evans and Jeske, 1993), ACMV (Etessami *et al.,* 1991), potato yellow mosaic virus (Sung and Coutts, 1995), TGMV (Elmer *et al.,* 1988; Sunter *et al.,* 1990; Sunter and Bisaro, 1992), and TYLCV-Sardinia (Laufs *et al.,* 1995) has shown that AC1 is the replication initiator protein essential for replication, that AC2 is a transcriptional activator of CP and BV1, and that AC3 is required for efficient DNA replication and infection. Our results with AC1, AC2, and AC3 mutants of ToLCV-India have confirmed these findings.

The mechanism by which the replication of ToLCV-India is regulated appears to be complex. Even though AV2 protein is not required for viral replication, mutations in AV2 that did not involve deletion reduced viral replication; CP synthesis was required for this effect. When the expression of AV2 and CP was disrupted in mutants M1te/R66fr and M19T/R66fr, replication was increased ~20-fold over M1te and M19T in protoplasts. Preventing potential initiation of AV2 at Met19, or causing premature termination of the protein at Tyr24, decreased the DNA accumulation of AV2 deletion mutant Δ121-137 by ~10-fold. However, a shorter version of AV2 protein (initiating at Met19) was not detected by Western blot analysis. It is unlikely that all these mutations interfered with DNA replication by affecting viral DNA structure that is needed for normal replication. Additional evidence for the complexity comes from cotransfection experiments (Table 3). Cotransfection of protoplasts with AV2 mutants and a plasmid containing expression cassettes encoding CP or AV2 and CP resulted in inhibition of AV2 mutant ssDNA accumulation. The same phenomenon was also observed when protoplasts were cotransfected with AV2 mutants and a plasmid containing mutated AV2 and CP expression cassette. In protoplasts cotransfected with AV2 mutants that did not involve deletion and an AC1 mutant (AC1M), only AC1M dsDNA was detected. This indicates that viral DNA without a mutation in AV2 replicates preferentially over viral DNA with a mutation in AV2.

In conclusion, we have shown a role for AV2 and CP ORF sequences in viral replication by studying different types of mutations. From these studies, we suggest that multiple mechanisms may account for the observed complexity in viral replication. We favor a hypothesis that a viral or host factor interacts with A-component virion-sense mRNA in regulating viral replication. Changes in virion-sense mRNA structure, or its capacity to be translated, may affect this interaction. Future experiments will address the nature of this interaction by precisely defin-

ing the AV2 and CP nucleotide sequences that are responsible for this regulation and by identifying the factor(s) that is involved.

ACKNOWLEDGMENTS

This publication was made possible through support provided by the Office of Agriculture, Bureau for Research and Development, U.S. Agency for International Development, under Cooperative Agreement DAN-4197-A-00-1126-00, and support provided by ORSTOM, Paris, France.

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