Mutagenesis in ORF AV2 affects viral replication in *Mungbean yellow mosaic India virus*

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Mungbean yellow mosaic India virus (MYMIV) is a whitefly-transmitted begomovirus with a bipartite genome. We investigate the functions of the MYMIV-AV2 protein, the open reading frame present upstream of the coat protein gene in DNA A component. The ability of MYMIV-AV2 mutants to replicate, spread and cause symptoms in legume hosts, blackgram, cowpea and French bean was analysed. Plants agroinoculated with mutants K73R, C86S and the double mutant C84S,C86S showed increase in severity of symptoms compared with the wild type. However, mutants W2S and H14Q,G15E caused marked attenuation of symptoms. While the double mutants C84S,C86S caused a 50-fold increase in double-stranded supercoiled and single-stranded DNA accumulation, the mutations W2S and H14Q,G15E showed a decrease in double-stranded supercoiled and single-stranded viral DNA accumulation. Because AV2 mutants affect the ratio between open circular and supercoiled DNA forms, we hypothesize that these mutations may modulate the functions of the replication initiation protein.

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1. Introduction

Yellow mosaic disease is one of the most devastating viral diseases of grain legumes in Southeast Asia. Its causative agents, the species *Mungbean yellow mosaic India virus* (MYMIV) and *Mungbean yellow mosaic virus* (MYMV), belong to the genus *Begomovirus* of the family Geminiviridae. The family is characterized by twinned *para icosahedral* virion particles encapsidating a circular single-stranded (ss) DNA genome that replicates in a rolling circular manner through double-stranded (ds) intermediates (Stanley *et al.* 2005). The members of Geminiviridae are differentiated into four genera (Fauquet *et al.* 2008), *Mastrevirus*, *Curtovirus*, *Topocuvirus* and *Begomovirus*, on the basis of their host range, insect vector and genome organization. The genus *Begomovirus* comprises whitefly-transmitted geminiviruses that infect dicotyledonous plants and have either a monopartite (DNA A) or bipartite (DNA A and DNA B) genome. DNA A encodes the proteins required for replication, transcription and encapsidation (Hanley-Bowdoin *et al.* 1999; Rojas *et al.* 2005). DNA B encodes the proteins required for intra- and intercellular movement.

Keywords. AV2; begomoviruses; legumes; yellow mosaic

Abbreviations used: ACMV, *Arican cassava mosaic virus*; ds, double-stranded; EACMCV, *East African cassava mosaic Cameroon virus*; MYMV, *Mungbean yellow mosaic virus*; MYMV-Vig, *Mungbean yellow mosaic virus – Vigna*; ORF, open reading frame; PKC, protein kinase C; PTGS, post-transcriptional gene silencing; PTR, partial head to tail repeat; PVX, *Potato virus X*; Ss, single-stranded; ToLCNDV, *Tomato leaf curl New Delhi virus*; TYLCV-Is, *Tomato yellow leaf curl virus* – Israel

The DNA A component of both monopartite and bipartite begomoviruses of the Old World differs from those of the New World (Harrison and Robinson 1999) in having an additional virion sense open reading frame (ORF) upstream of the coat protein gene (ORF AV1/V1). This ORF designated as ORF AV2 in bipartite and V2 in monopartite begomoviruses is also referred to as pre-coat protein (Padidam *et al.* 1996). An ORF is present in the analogous position in leafhopper-transmitted mastreviruses (ORF V1) and curtoviruses (ORF V2). In general, pre-coat protein ORFs are not highly conserved between genera and the species within a genus. The percentage identity in amino acid sequence of AV2/V2 protein among Old World begomoviruses ranges from 45% to 60% (Padidam *et al.* 1996).

The exact role of ORF AV2/V2 is yet to be understood. The ORF V1 in mastreviruses (Dickinson *et al.* 1996; Boulton *et al.* 1989, 1993) and V2 in monopartite begomoviruses (Rigden *et al.* 1993; Wartig *et al.* 1997; Rojas *et al.* 2001; Gafni and Epel 2002) were found to be responsible for cell-to-cell spread of the virus. Apart from its role in cell-to-cell spread, ORF V2 in curtoviruses was demonstrated to modulate levels of ds and ss viral DNA (Stanley *et al.* 1992; Hormuzdi and Bisaro 1993). Its role as the suppressor of RNA silencing was shown for a begomovirus, *Tomato yellow leaf curl virus* – Israel, *i.e.* TYLCV-Is (Zrachya *et al.* 2007; Glick *et al.* 2008).

The role of ORF AV2 in Old World bipartite begomoviruses is puzzling as the movement function is facilitated by BV1 and BC1 proteins encoded by DNA B component. The role of ORF AV2 in cell-to-cell trafficking has been demonstrated in two bipartite begomoviruses *Tomato leaf curl New Delhi virus* (Padidam *et al.* 1996) and in *Indian cassava mosaic virus* (Rothenstein *et al.* 2007). The ability of AV2 of another bipartite begomovirus, *East African cassava mosaic Cameroon virus* (EACMCV), was shown to suppress viral-induced gene silencing when both inducer and suppressor were expressed from a *Potato virus X* (PVX) vector (Chowda-Reddy *et al.* 2008). This activity involved a putative protein kinase C (PKC) phosphorylation motif.

In this article, we will elucidate the functions of ORF AV2 of MYMIV. Site-directed mutagenesis of MYMIV-AV2 was performed to determine whether ORF AV2 had a role in viral DNA accumulation and symptom production.

2. Materials and methods

2.1 *Virus isolates*

The role of ORF AV2 was investigated for blackgram (Bg) and cowpea (Cp) isolates of *Mungbean yellow mosaic India virus* (MYMIV-Bg3 – GenBank accession numbers: AF126406 and AF142440; Mandal *et al.* 1997; MYMIV-Cp – GenBank accession numbers: AF481865 and AF503580; Malathi *et al.* 2005). These two isolates were chosen as they showed 51% variability in ORF AV2 (figures 1A and B) (Surendranath *et al.* 2005). The difference between the two isolates is essentially due to a naturally occurring insertional mutation (A at nucleotide coordinate 336). The clones pBgH13 and pCpH2 representing DNA A components of Bg and Cp isolates were used to generate mutation; the clones pBgB12 and pCpB3 representing DNA B components of Bg and Cp isolates, respectively, were used for infectivity studies.

2.2 *Site-directed mutagenesis*

Mutagenesis of ORF AV2 was carried out using a quick-change TM-site directed mutagenesis kit (Stratagene) by following the manufacturer's protocol. The description of the amino acid substitution and the sequence of oligonucleotides used to generate mutations are given in tables 1 and 2. Amino acid substitutions were carried out by targeting the most conserved amino acids (W2, K73, C84 and C86) or the amino acids in the ORF AV2 present only in MYMIV isolates. The primers contained the desired substitution or insertion in the middle, and mutations were confirmed by the sequence analysis of a minimum of eight transformants.

ORF AV2 encodes a putative protein of 74 amino acids in the Bg isolate (figure 1B) and 113 amino acids in the Cp isolate; in the mutation ΔATG, the start codon ATG is altered to ATC and this alteration is expected to give rise to a truncated AV2 protein with N′ terminal deletion of 18 amino acids, being initiated from a downstream ATG.

Mutation BgdelA was introduced only in the Bg isolate; multiple alignment of the nucleotide and amino acid sequence of ORF AV2 of Bg and Cp isolates revealed an insertion of 'A' at nucleotide coordinate 336, resulting in truncation of the putative protein in the Bg isolate. BgdelA mutation was made at this site to restore the full ORF.

In total, five mutations in the Bg isolate and seven in the Cp isolate were made. The mutations H14Q,G15E and C84S,C86S were double mutations. In the case of the Cp isolate, ORF AV2 overlaps with ORF AV1. The mutations introduced after overlapping regions were K73R, C86S and C84S,C86S. The expression strategy of these overlapping ORFs is not clear for this virus as both a longer transcript encompassing ORF AV1 and AV2 and a shorter transcript from which only ORF AV1 may be translated have been identified (Usharani *et al.* 2006). Change of amino acid lysine to arginine (K73R) in ORF AV2 led to change of R at the 19th position to G (arginine to glycine) in ORF AV1; in the other two mutations C86S and C84S,C86S, there was no change in any amino acid residues in ORF AV1.

2.3 *Viral constructs for infectivity*

Partial head to tail repeat (PTR) constructs of viral genomic components were made as given below. The recombinant

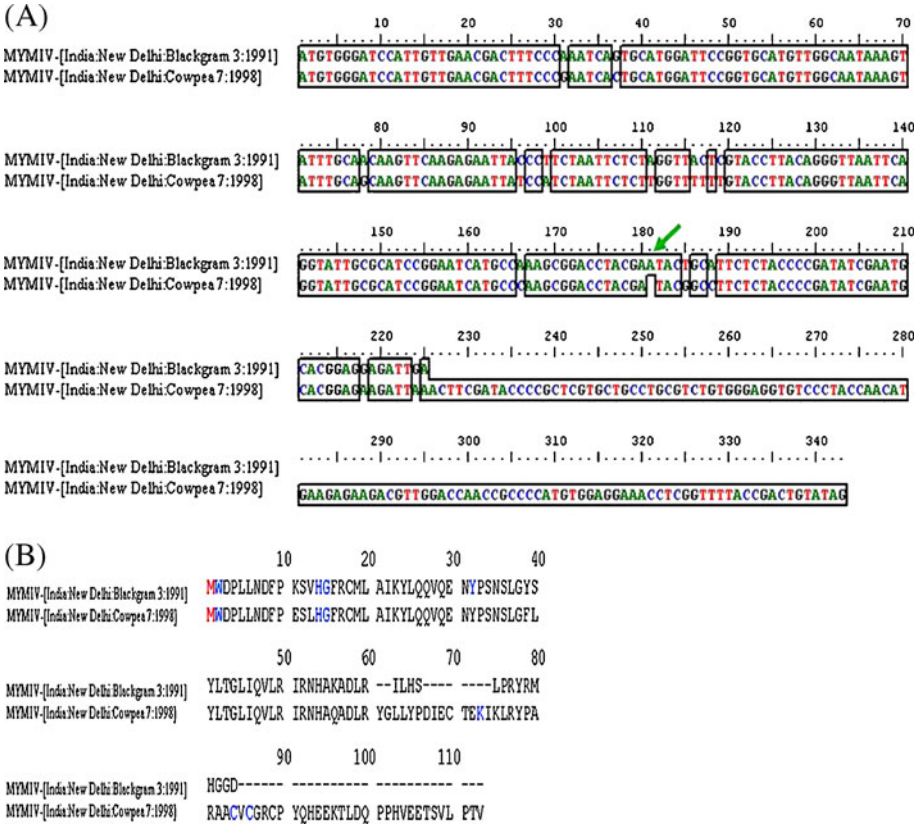

Figure 1. (**A**) Pairwise alignments of the nucleotide sequences of the ORF AV2 of Bg and Cp isolates. (**B**) Pairwise alignment of the predicted amino acid sequences of the AV2 protein of Bg and Cp isolates. Dashes indicate gaps introduced for maximum alignment. Mutation sites are indicated in colour.

DNA A clones pBgH13 (Bg isolate) and pCpH2 (Cp isolate) were restricted with *Hin*dIII/*Kpn*I to release a 1.8 kb fragment, which was cloned in the vector pBin19 to give pBinBgA (0.6 mer) or pBinpCpA (0.6 mer). A unit-genome-length DNA A component in pBgH13 and pCpH2 was released by *Hin*dIII/*Bgl*I restriction and the gel-eluted DNA A by a 2.7 kb fragment with *Hin*dIII ends was ligated to the dephosphorylated *Hin*dIII ends of pBinBgA (0.6 mer)

Table 1. Primers used for site directed mutagenesis of ORF AV2 in blackgram isolate of MYMIV-[India:New Delhi:Blackgram 3:1991]

Mutant	Orientation	Primer sequence	Mutation site	Nucleotide co-ordinates	Position of amino acid substitution
ΔATG	FP	5′-AACCCACTAACAAT***C***TGGGATCCATTG-3′	ATG to ATC	144-170	1
	RP	5′-CAATGGATGCCA***G***ATTGTTACTGGGTT-3	Met to ILe		
BgdelA	FP	5′-GCGGACCTACGA↓TACTGCATTCTAC-3′	Deletion A	324-351	61
	RP	5′-GTGAGAATGCAGT↓ATACGRAGGTCGC-3′			
W2S	FP	5′-CACTAACAATGT***C***GGATCCATTGTTG-3′	TGG to TCG	148-173	2
	RP	5′-CCACAATGGATCC***G***ACATTGTTAGTG-3′	Trp to Ser		
H14Q,G15E	FP	5′-CCAAATCAGTGCA***A***GA***A***TTCCGGTGCATG-3′	CAT to CAA (His to Gln)	183-212	14 and 15
	RP	5′-CAGCACCGGAAT***T***CT***T***GCACTGATTTGG-3′	GGA to GAA (Gly to Glu)		
Y32F	FP	5′-GTTCAAGAGAATT***T***CCCTTCTAATTC-3′	TAC to TTC	237-282	32
	RP	5′GAATTAGAAGGG***A***AATTCTCTTGAAC-3′	Try to Phe		

The nucleotide changed is shown in italic bold. FP: Forward primer (viral sense), RP: Reverse primer (complementary sense).

Table 2. Primers used for site directed mutagenesis of ORF AV2 in cowpea isolate of MYMIV-[India:New Delhi:Cowpea 7:1998]

Mutant	Orientation	Primer sequence	Mutation site	Nucleotide co-ordinates	Position of amino acid substitution
ΔATG	FP	5′-CCCAGTAACAAT***C***TGGGATCCATTG-3′	ATG to ATC	146-170	1
	RP	5′-CAATGGATGCCA***G***ATTGTTACTGGG-3′	Met to ILe		
W2S	FP	5′-CAGTAACAATGT***C***GGATCCATTGTTG-3′	TGG to TCG	148-173	2
	RP	5′-CAACAATGGATCC***G***ACATTGTTACTG-3′	Trp to Ser		
H14Q,G15E	FP	5′-CCGAATCACTGCA***A***G***A***ATTCCGGTGCATG-3′	His to Gln (CAT to CAA)	184-212	14 and 15
	RP	5′-CATGCACCGGAAT***T***C***T***TGCAGTCATTCGG-3′	GGA to GAA (Gly to Glu)		
K73R	FP	5′-TGCACGGAG***AG***GATTAAACTT-3′	AAG to AGG	363-383	73
	RP	5′-AAGTTTAATC***CT***CTCCGTGCA-3′	Lys to Arg		
C86S	FP	5′-TGCCTGCGTC***A***GTGGGAGGTGT-3′	TGT to AGT	401-422	86
	RP	5′-ACACCTCCCAC***T***GACGCAGGCA-3′	Cys to Ser		
C84S,86 S	FP	5′-TGCTGCC***A***GCGTC***A***GTGGGA-3′	TGC to AGC (Cys to Ser)	398-417	84 and 86
	RP	5′-TCCCAC***T***GACGC***T***GGCAGCA-3′	TGT to AGT (Cys to Ser)		
G44N	FP	5′-GTACCTTACAG***A***CTTAATTCAGG-3	GGG to GAC	275-200	44
	RP	5′-CCTGAATTAAG***T***CTGTAAGGTAC-3′	Gly to Asp		

The nucleotide changed is shown in italic bold. FP: Forward primer (viral sense), RP: Reverse primer (complementary sense).

and pBinCpA (0.6 mer) to give 1.6 mer of DNA A component. Mutations were performed using plasmid pBgH13 and pCpH2, and after confirmation, by sequencing, the full-length genome with mutation was released by *Hin*dIII/*Bgl*I restriction and ligated to pBinBgA (0.6 mer) or pBinCpA (0.6 mer) to give pBinBg (1.6 mer) and pBinCp (1.6 mer) of AV2 mutants. Dimeric constructs of DNA B components were made as by Mandal *et al.* (1997) and Malathi *et al.* (2005).

2.4 *Agroinoculation*

For agroinoculation, the selected PTR constructs were mobilized into *Agrobacterium tumefaciens* strain EHA 105 by triparental mating. Agroinoculations were carried out by the sprout-seed method (Mandal *et al.* 1997). The seeds of blackgram *Vigna mungo* cv. T9, cowpea *V. unguiculata* cv. Pusa Komal and French bean *Phascolus vulgaris* cv. Sel-9 were procured from National Seeds Corporation, India. Plants were maintained for 30 days in National Phytotron Facility at 27±2°C, RH 85, day light 18000 lux.

2.5 *Southern blot analysis*

Genomic DNA was extracted by Gem-CTAB method (Rouhibakhsh *et al*. 2008) from the third trifoliate leaves of systemically infected plants 21 days post inoculation. Leaves from three plants were pooled as one sample and analysed for every mutation–host combination. Total nucleic acid (5 μg) was electrophoresed in 1.2% agarose gel and transferred to a nylon membrane, and viral DNA was detected by using coat protein or movement protein gene fragments as a radiolabelled probe. In order to determine the nature of the viral replicative form, viral DNA from wild-type inoculated plants were treated with S1 nuclease and exonuclease III, and analysed by Southern blots. Viral DNA forms were identified based on the initial observations. The increase and decrease in replicative forms of viral DNA were assessed by comparing viral replicative forms extracted from plants inoculated with the wild type. The quantification of each viral DNA form was done by comparing it everytime with the respective wild type by using the Manual Band Quantification programme of Gene Tools (ver 3.06) from Syngene, Cambridge, England.

2.6 *Immunosorbent electron microscopy*

ORF AV2 overlaps with the coat protein ORF AV1. To confirm that the mutations in AV2 have not anyway interfered with coat protein expression, geminate particles were visualized by immunosorbent electron microscopy. The method followed was as described by Roberts *et al.* (1984). Carbon-coated grids were floated over a 10 μl drop of 1:1000 diluted *Arican cassava mosaic virus* (ACMV)

Table 3. Infectivity of wild type and mutants of the Bg isolate of MYMIV on legume hosts by agroinoculation

Virus isolates	Host	No. of plants infected/No. of plant inoculated		Average infectivity (%)	Symptoms
		Expt.1	Expt. 2		
*BgA + BgB	Blackgram	14/42	11/34	32.8	YM
	Cowpea	12/34	5/18	32.95	St, MLC, LD, PK
	French bean	33/33	16/16	100	St, DLR, LC, PK, D
ΔATG	Blackgram	15/35	13/32	41.7	YM
	Cowpea	11/31	5/19	30.85	St, MLC, LD, PK, RYM
	French bean	36/36	20/20	100	St, DLR, LC, PK, D
Bg delA + BgB	Blackgram	18/30	21/33	61.8	YM
	Cowpea	14/35	7/18	39.4	St, MYM, MLC
	French bean	30/30	16/16	100	St, DLR, PK, D
W2S	Blackgram	17/38	13/36	44	RYM
	Cowpea	16/33	7/15	47.5	St, MLC
	French bean	32/32	14/14	100	St, DLR,PK
H14Q,G15E	Blackgram	14/19	17/24	74.8	AYM
	Cowpea	16/42	6/17	36.65	MLC
	French bean	29/32	15/16	91.8	St, LC
Y32F	Blackgram	16/29	18/33	56.35	YM
	Cowpea	12/33	5/15	34.8	MLC, RYM
	French bean	29/29	15/15	100	St, LC

St: Stunting; DLR: Downward leaf rolling; LC: Leaf Curling; PR: Puckering; D: Death; MLC: Mild leaf curl; LD: Leaf deformation; YM: Yellow mosaic: MYL: mild Yellow Mosaic; RYM: Restricted Yellow Mosaic; AYM: attenuated yellow mosaic.
*Wild type.

antibody in phosphate buffer (pH 6.5, 0.07 M) for 1 h at 37°C. Excess antibodies were drained off, grids were washed and antibody-coated grids were floated on a leaf dip preparation of leaf samples and stained with 2% uranyl acetate solution. The third trifoliate leaves from five French bean plants were examined for wild types and mutants. For every mutation, five grids were scanned and the approximate numbers of typical geminate particles per field observed were counted.

3. Results

3.1 *Infectivity and symptom production in wild type and AV2 mutants*

In general, all the AV2 mutants of Bg and Cp isolates were as infectious as the wild type and induced symptoms (tables 3 and 4) within 7–15 days post inoculation. In plants inoculated with mutants, the severity of symptoms was marginally affected in blackgram vis-à-vis cowpea and French beans, in which symptom attenuation was very prominent. There were marked differences in symptom phenotypes.

In the case of Bg isolates, the wild type produced small, well-defined squarish yellow mosaic areas constricted by vascular tissue in blackgram. Unlike in the wild type, in plants inoculated by W2S, H14Q,G15E and Y32F mutants, the chlorotic area in leaf lamina was not well defined; they were diffused or scattered (figure 2C) and appeared as yellow chlorotic blotches alternating with green areas, which differed from yellow mosaic symptoms by having definite outlines.

In the case of cowpea, which is not a well-adapted host for Bg isolates, atypical symptoms of leaf curl seen in the wild type was also seen in the mutants. The mutant BgdelA was predicted to restore the full-length AV2 protein, and the presence of full-length AV2 protein was expected to facilitate infection of cowpea by the Bg isolate. However, this mutant produced leaf curl and asymmetrical leaflet phenotype similar to the wild-type Bg isolate on cowpea, indicating the absence of AV2 protein's role in yellow mosaic symptom expression. There was no yellow mosaic or chlorotic areas, and the isolate continued to be less adapted to cowpea as the wild type. In the plants inoculated by mutants, leaf curl was mild, stunting was less and there was asymmetry between the leaflets of the trifoliate leaf. Attenuation of symptoms was more prominent in the double mutant H14Q,G15E; in the mutant Y32F, as against the wild type, chlorotic area appeared along with leaf curl.

Table 4. Infectivity of wild type and mutants of the Cp isolate of MYMIV on legume hosts by agroinoculation

Virus isolates	Host	No. of plants infected/No. of plant inoculated		Average infectivity (%)	Symptoms
		Expt.1	Expt. 2		
*CpA + CpB	Blackgram	13/27	23/49	47.3	YM
	Cowpea	20/31	17/28	62.55	GM
	French bean	29/29	25/25	100	St, RYM, DLC
ΔATG	Blackgram	17/29	18/32	57.8	YM
	Cowpea	13/31	9/23	40.5	YM
	French bean	22/22	14/14	100	St, LC, PK
W2S	Blackgram	7/21	8/21	34.95	RYM
	Cowpea	13/27	9/17	50.5	RYM
	French bean	24/24	12/12	100	LC, PK, RYM
H14Q,G15E	Blackgram	13/25	17/34	51	AYM
	Cowpea	18/27	9/14	65.4	AYM
	French bean	27/27	11/12	95.5	YM, St, MLC
K73R	Blackgram	9/29	9/36	30.5	YM
	Cowpea	13/20	10/16	63.75	St, GM, VC, MLC
	French bean	32/32	13/13	100	St, YM, MLC, PK
C86S	Blackgram	7/21	10/29	33.85	YM
	Cowpea	21/34	9/16	58.95	YM, VC
	French bean	12/12	13/13	100	St, RYM, DLC
C84S,86S	Blackgram	8/23	9/28	33.4	RYM
	Cowpea	12/19	8/3	62.3	YM, VC
	French bean	25//25	12/12	100	RYM, MLC
G44N	Blackgram	8/25	8/25	32	YM
	Cowpea	22/35	8/13	62.15	YM, MLC
	French bean	17/20	14/14	92.5	RYM, St, DLC

St: Stunting; RYM: Restricted yellow mosaic; DLC: Downward leaf curling; YM: yellow mosaic; GM: Golden mosaic; VC: Vein clearing; MLC: Mild leaf curl; AYM: attenuated yellow mosaic; PK: puckering.
*Wild type.

AV2 mutations severely affected symptoms in French bean; the wild-type Bg isolate produced severe stunting, downward curling of cotyledonary leaf (figure 2A) and the plant remained stunted without any further growth. The first trifoliate leaf, if produced, was very small and showed puckering. However, in the case of mutants W2S and H14Q,G15E, the symptoms were mild, elongation of the shoot was normal and new trifoliate leaves appeared. The leaves were of normal size and did not show puckering.

When inoculated with the Cp isolate, the blackgram plants showed well-distributed yellow mosaic pattern throughout the lamina, comparable to the wild type in ΔATG, K73R, C86S and C84S,C86S mutants (figure 2C). In two mutations, W2S and H14Q,G15E, very sparse, small, squarish chlorotic areas were seen, and symptoms were almost not visible in ~50% of the plants.

Changes in symptoms were more pronounced in cowpea plants. In both W2S and H14Q,G15E, symptoms were mild – only small restricted yellow mosaic areas were seen, which did not advance to golden mosaic. In mutants K73R, C86S and C84S,C86S, veinlets showed yellowing (figure 2B), which spread to major veins, resulting in a prominent yellow vein mosaic symptom. In the mutant C84S,C86S, the symptoms were more severe than in the wild type. In G44N, uniformly distributed yellow mosaic pattern, asymmetry in leaflets and slight puckering of the leaflet were seen instead of golden mosaic of the whole lamina.

In French bean, inoculation with the mutants W2S and H14Q,G15 produced attenuated symptoms. Instead of extreme stunting, normal-length shoots and emergence of trifoliate leaves were seen. Interesting results were seen with mutants K73R, C86S and C84S,C86S – small chlorotic areas leading to yellow mosaic, puckering and downward leaf curl symptoms were observed. This change in the symptom is very significant, as MYMIV isolates till date are

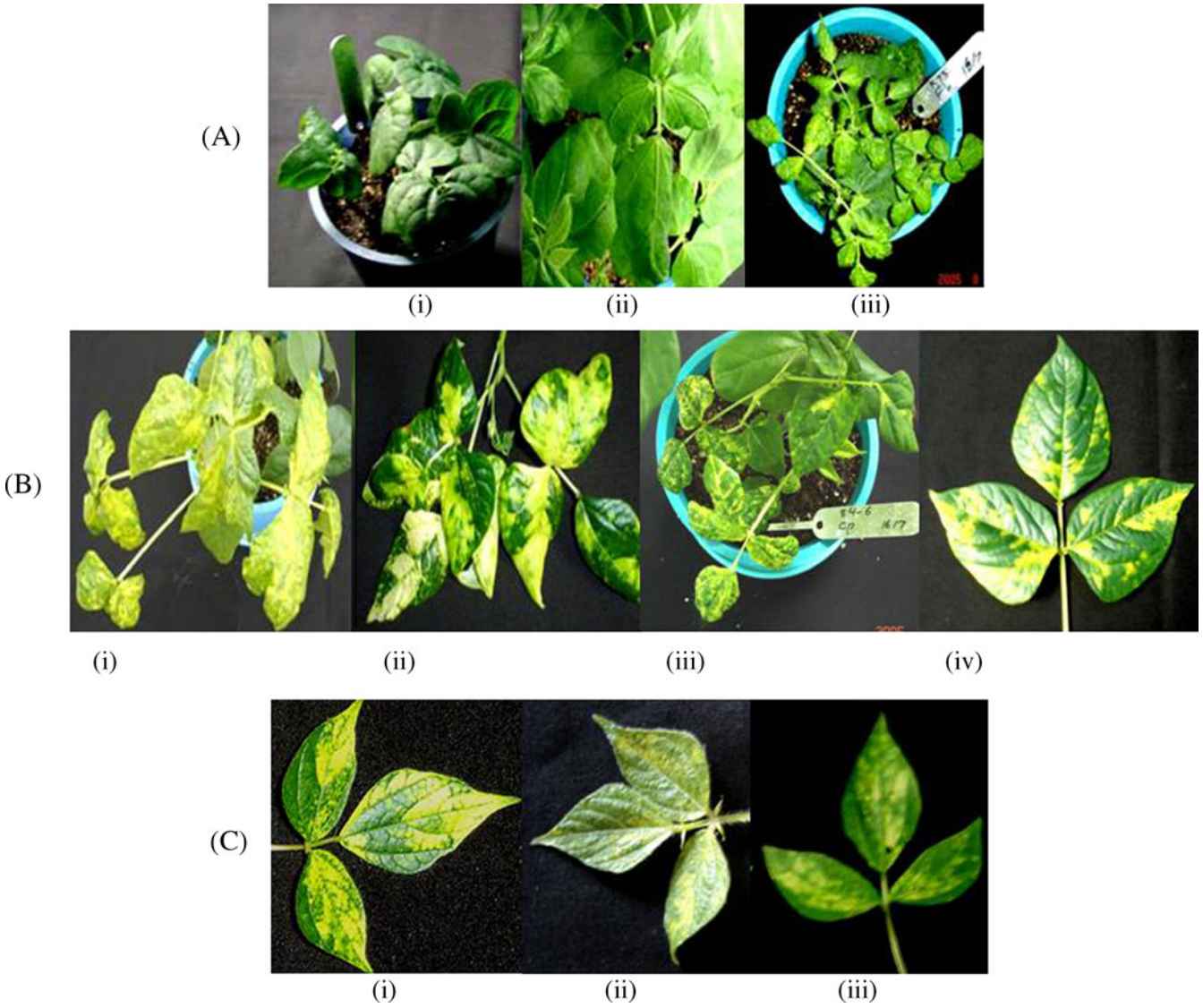

Figure 2. (**A**) French bean (cv Sel 19) plants showing symptoms after agroinoculation with wild type and mutants of the Bg isolate and Cp isolate: (i) downward leaf curling and stunting in wild type, (ii) attenuation of symptoms in the mutant H14Q,G15E and (iii) yellow mosaic downward leaf curling in the mutant K73R. (**B**) Cowpea (cv. Pusa Komal) plants showing symptoms after agroinoculation with wild type and mutants of the Cp isolate: (i) golden yellow mosaic by wild type, (ii) restricted yellow mosaic with puckering in mutant K73R, (iii) veinal yellowing in the mutant C84S,C86S and (iv) attenuation of symptoms in the mutant H14Q,G15E. (**C**) Blackgram plants showing symptoms after agroinoculation with wild type and mutants of the Bg isolate and Cp isolate: (i) severe yellow mosaic by wild type, (ii) attenuation of symptoms in the mutant H14Q,G15E and (iii) very faint yellow symptoms in the mutant ΔATG.

known to produce only leaf curl, stunting and puckering in French bean, and not yellow mosaic.

3.2 *Viral replication*

In Southern blot analysis, the double-stranded (ds), open circular (oc), linear (lin), supercoiled (sc) and single-stranded (ss) forms were distinguished, on the basis of resistance to *Exonuclease III* and susceptibility to *S1 nuclease* treatment. Of the two faster migrating bands running on par with 1.6 kb and 1.0 kb fragments of the DNA molecular weight marker, the slower one (~1.6 kb) was identified to represent the ds sc viral DNA form and the faster one, the ss DNA form. In cowpea inoculated by the wild-type Bg isolate or its mutants, viral DNA replicative forms were very few. These forms were not clearly visible even in blots prepared with 20 μg of total

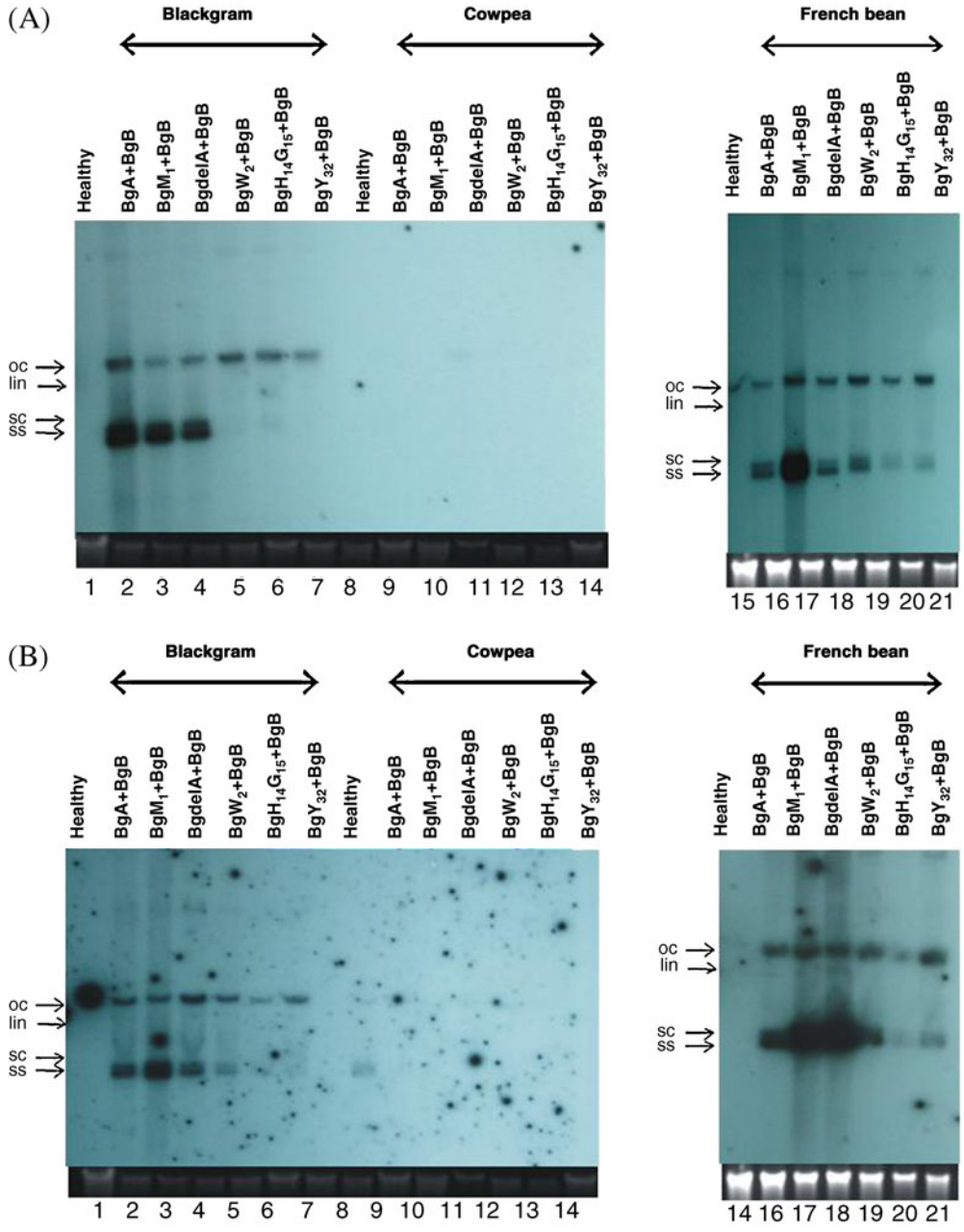

Figure 3. Southern blot analysis of viral replicative forms in legume host, agroinoculated with the Bg isolate. (**A**) Radiolabelled coat protein gene was used as probe. (**B**) Radiolabelled movement protein gene was used as probe. oc: double stranded open circular; lin: double stranded linear; sc: double stranded supercoiled; ss: single stranded.

nucleic acid. However, the presence of viral DNA in cowpea plants was verified by PCR, using abutting primers that generate a 2.7 kb full-length genome. PCR amplicons were obtained in all the samples, confirming viral DNA presence. The undetectable level of viral DNA was also seen in plants inoculated by the mutant BgdelA, indicating that restoration of the frame did not lead to better adaptation of the Bg isolate to this host. Except this Cp/Bg isolate combination, in all the samples of blackgram, French bean and cowpea inoculated by wild type and AV2 mutants, viral replicative DNA forms (figures 3 and 4) were present. Accumulation of ds, sc and ss DNA levels either increased or decreased than when inoculated with the wild type. From the results it is evident that mutations in AV2 were not detrimental to

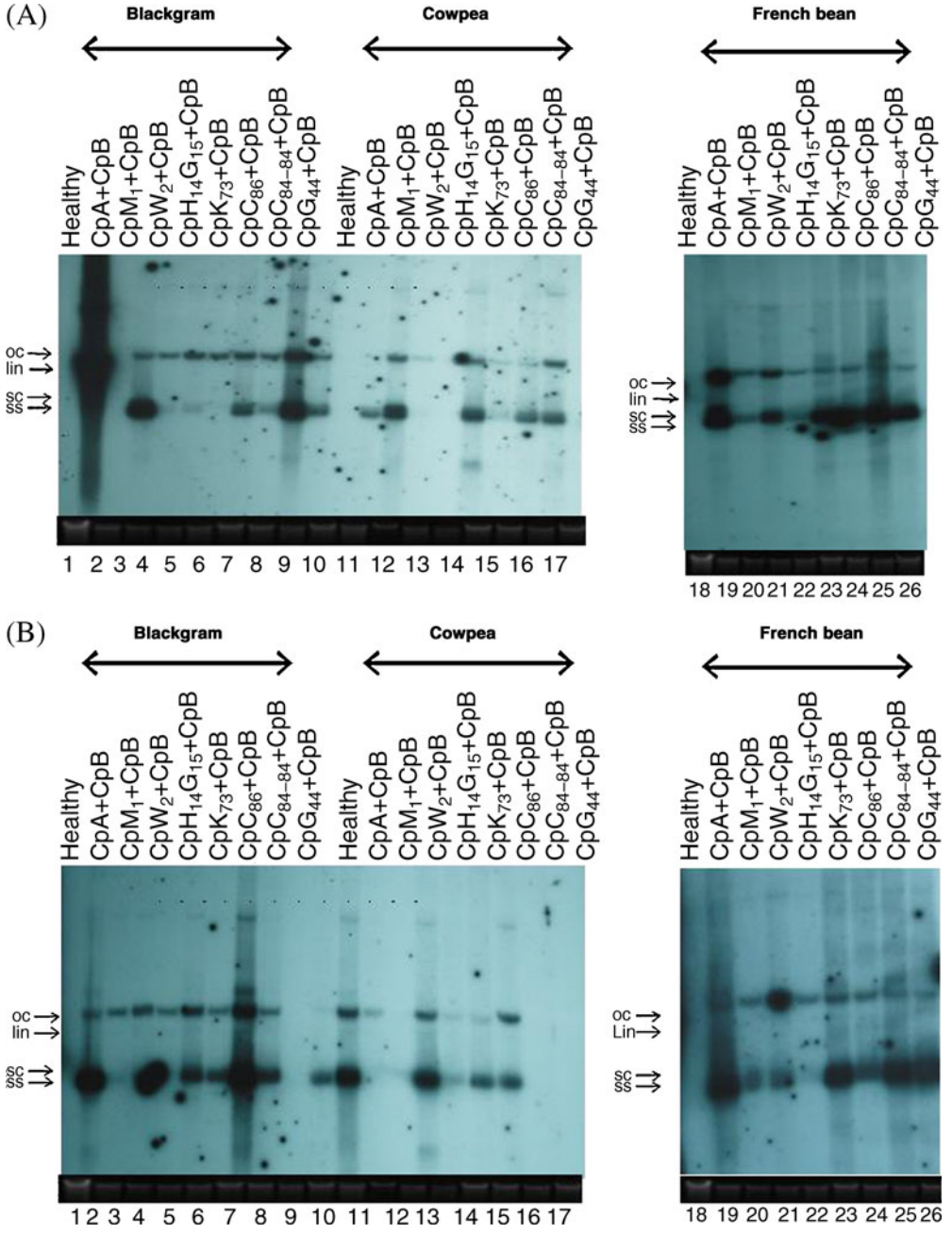

Figure 4. Southern blot analysis of viral replicative forms in legume host, agroinoculated with the Cp isolate. (**A**) Radiolabelled coat protein gene was used as probe. (**B**) Radiolabelled movement protein gene was used as probe. oc: double stranded open circular; lin: double stranded linear; sc: double stranded supercoiled; ss: single stranded.

replication although they strongly affected the ratio between levels of ds and ss DNA.

Significantly, the mutations W2S and H14Q,G15E showed reduction in sc and ss DNA levels in all the three hosts. Similar decrease was also seen in DNA B component. The reduction in sc and ss DNA ranged from 70% to 90% compared with the wild-type infection. Significant results were seen with the four mutants K73S, G44N, C86S and C84S,C86S. In blackgram, which showed increase in ds oc (up to 100%), linear and ss DNA levels were either on par with the wild type or were reduced to 53%. In blackgram, a similar increase was also seen with K73S, C84S,C86S and G44N mutants for cowpea.

Surprisingly, in French bean, ds sc and ss DNA levels remained more or less comparable to the wild type, but the ds oc level was considerably reduced (up to 74%).

Typical geminate particles were observed in all the plants inoculated by wild type or mutants, indicating that ORF AV1 expression is not affected by mutation of ORF AV2.

For every host–mutant combination, the DNA extracted from three plants was PCR-amplified and the sequence around the mutation was determined. Reversion to wild type was seen only in the case of the mutant ΔATG. The sequence ATC reverted to ATG, restoring the gene, in two of the three products of blackgram and French bean samples infected by Bg and Cp isolates. In all the other cases the original mutation was found to be retained.

4. Discussion

In the present study, the role of ORF AV2 of MYMIV in viral replication and symptom development was analysed by using site-directed mutagenesis. Transcription strategy of viral sense genes (ORF AV2 and ORF AV1) of YMV isolates have been studied for one isolate, each of MYMV and MYMIV. Shivaprasad *et al.* (2005) suggested that ORF AV2 and AV1 of *Mungbean yellow mosaic virus – Vigna* (MYMV-Vig) are translated from a discistronic transcript, which has two different 5′ transcription start sites – one at 4 nt above ATG of ORF AV2 and the other at ATG of AV2 ORF. Usharani *et al.* (2006) reported two different transcription start sites for viral sense ORFs of MYMIV-Bg – one, at 4 nt upstream of AV2 ATG, similar to MYMV, and the other at 104 nt upstream of ATG of ORF AV1; this is suggestive of both monocistronic and discistronic transcripts for viral sense genes. As the expression strategy for these ORFs is yet to be resolved, to study the function of ORF AV2, mutations were planned in such a way that the coat protein ORF AV1 was not affected. Five mutations planned for the Bg isolate did not affect Cp synthesis, because in this case AV2 ORF ends prematurely and does not overlap with AV1. In the case of the Cp isolate, ORF AV2 overlaps with ORF AV1. Of the three mutations K73R, C86S and C84S,C86S, only mutation K73R resulted in change of amino acid of coat protein: the amino acid residue R at 19th position was changed to G and other amino acid residues were not affected. From immunosorbent electron microscopy studies, which revealed typical geminate particles, it is also clear that ORF AV1 is not affected and is expressed well. Therefore, symptom phenotype changes observed in the mutants are justifiably attributed to alteration in AV2 protein.

When the ΔATG mutant was inoculated, most of the plants developed wild-type infection. The replicative forms were also on par with the wild type. Sequencing of PCR products revealed reversion of mutation restoring ORF AV2. Such reversions to wild-type V2 have been observed in other begomoviruses (Wartig *et al.* 1997; Rigden *et al.* 1993)

In all the experiments conducted on three host species, the wild type and AV2 mutants could infect, systemically spread and cause symptoms. The mutants differed from the wild type only in symptom phenotype. Symptoms were attenuated and yet distinguishable from uninoculated healthy plants in the case of mutant W2S and H14Q,G15E; a symptom phenotype of yellow vein mosaic as against wild-type golden mosaic was seen in mutants K73R, C86S and C84S,C86S in cowpea. The most significant change observed was yellow mosaic symptom produced in French bean by the mutants Y32F and K73R as against downward leaf curl and puckering expressed by the wild type. Systemic spread of none of the mutants was affected. In the studies conducted with monopartite begomoviruses, *Tomato leaf curl virus* – Australia (Rigden *et al.* 1993), *Tomato yellow leaf curl virus* – Sardinia (Wartig *et al.* 1997) and *Tomato yellow leaf curl virus* – Israel (Zrachya *et al.* 2007), disruption of V2 did not affect the systemic spread of the virus. In the bipartite begomoviruses *Tomato leaf curl New Delhi virus* (ToLCNDV), too, mutation in AV2 alone did not affect systemic spread of the virus (Padidam *et al.* 1996).

However, in all the cases cited above, inoculated plants showed either attenuated symptoms (ToLCNDV; Padidam *et al.* 1996) or were symptomless (Rigden *et al.* 1993; Wartig *et al.* 1997). In the present work, the results of the double mutation C84S,C86S expressing more severe symptoms than the wild type is especially contrasting to the work of Padidam *et al.* (1996), who observed only mild chlorosis and mild leaf curl symptoms. Based on micro-injection of V2-GFP fusion construct, in TYLCV-Is, Gafni and Epel (2002) suggested that V2 modulates Cp-mediated nuclear export of viral DNA for cell-to-cell movement through the mesophyll plasmodesmata. Interestingly, in a bipartite begomovirus *Indian Cassava mosaic virus*, intercellular movement of AV2-GFP in tissues other than phloem (Rothenstein *et al.* 2007) was shown. In light of the above evidence of AV2-mediated trafficking of viral DNA from the nucleus to the cell periphery and to the adjoining cell, the change in symptom phenotype assumes importance. The characteristic yellow mosaic symptom changing to yellow vein mosaic type seen here is suggestive of the role of the AV2 mutant in facilitating the spread of viral DNA to the surrounding tissue outside the phloem.

Altered levels of viral DNA may occur either due to reduced replication or systemic movement. In the present analysis, viral DNA was isolated from the third trifoliate leaf of infected plants 21 days post inoculation. Double-stranded oc forms were seen in all the mutants except the Bg isolate on cowpea, wherein even wild-type replicative forms were few. Wartig *et al.* (1997) compared the V1 protein of TYLCV with the M13 gene 5 protein (Kornberg and Baker 1992), which interferes with synthesis of duplex replicative

forms and prepares DNA for packaging in the virus particle. Padidam *et al.* (1996) observed reduced levels of viral DNA in *Nicotiana benthamiana* plants inoculated with AV2 mutants of ToLCNDV, and especially in the mutation C84S,C86S, the reduction compared with the wild type was 18% for ss DNA and 46% for ds DNA. However, in the present study on MYMIV, there was increase in ds oc DNA (up to 63%) over the wild-type infection for this mutation.

The amino acid residues C86 and C84 are predicted to govern oligomerization (Padidam *et al.* 1996). In our study, inhibition of oligomerization by altering both cysteine residues enhanced the replication level, contrasting with the single mutation of C86S, in which generation of ds sc DNA and ss DNA level is affected. The C84S,C86S mutation in a monopartite begomovirus, TYLCV-Is, lost its ability to interact with the tomato analogue of the *Arabidopsis* SGS3 protein, which is known to be involved in the RNA silencing pathway. The mutant, however, showed attenuated symptoms, which were attributed to the loss of post-transcriptional gene silencing (PTGS) activity (Zrachya *et al.* 2007; Glick *et al.* 2008). In our study, the double mutation enhanced symptom severity, altered symptom phenotype and resulted in enhanced replication level, in contrast to its predicted role as a PTGS suppressor. We hypothesize that in MYMIV, the AV2 protein may modulate the activity of replication initiation protein; this may be linked to the oligomerization domain of C84S,C86S. How it results in enhanced viral DNA needs to be looked into. Chowda-Reddy *et al*. (2008) discussed the PTGS activity of AV2 protein for an Old World bipartite begomovirus, *East African cassava mosaic Cameroon virus* (EACMCV), and attributed the activity to a phosphorylation motif of a putative protein kinase C (PKC). In a multiple alignment of predicted amino acid of AV2 protein of yellow mosaic viruses, such a motif was not seen.

From this study, it appears that the ORF AV2 protein may have a role in symptom development and regulating viral DNA accumulation in MYMIV; whether there is a difference in the role of AV2 protein among Old World bipartite begomoviruses needs to be further investigated.

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