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ORIGINAL ARTICLE

Expression of full-length and truncated Rep genes from Mungbean yellow mosaic virus-Vigna inhibits viral replication in transgenic tobacco

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Abstract Mungbean yellow mosaic virus-Vigna (MYMV-Vig) is a bipartite geminivirus that causes a severe yellow mosaic disease in blackgram. An assay was developed to study MYMV-Vig replication by agroinoculation of tobacco leaf discs with partial dimers of the virus. This assay, in a non-host model plant, was used to evaluate pathogen-derived resistance contributed by MYMV-Vig genes in transgenic plants. Viral DNA accumulation was optimum in tobacco leaf discs cultured for 10 days after infection with *Agrobacterium tumefaciens* strain Ach5 containing partial dimers of both DNA A and DNA B of MYMV-Vig. Transgenic tobacco plants with MYMV-Vig genes for coat protein (CP), replication-associated protein (Rep)-sense, Rep-antisense, truncated Rep (T-Rep), nuclear shuttle protein (NSP) and movement protein (MP) were generated. Leaf discs from transgenic tobacco plants, harbouring MYMV-Vig genes, were agroinoculated with partial dimers of MYMV-Vig and analyzed for viral DNA accumulation. The leaf discs from transgenic tobacco plants harbouring CP and MP genes supported the accumulation of higher levels of MYMV-Vig DNA. However, MYMV-Vig accumulation was inhibited in one transgenic plant harbouring the Rep-sense gene and in two plants harbouring the T-Rep gene. Northern analysis of these plants revealed a good correlation between expression of Rep or T-Rep genes and inhibition of MYMV-Vig accumulation.

Keywords Agroinfection · Geminivirus · *Mungbean yellow mosaic virus* · Pathogen-derived resistance · Replication-associated protein · Truncated Rep

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Introduction

Geminiviruses comprise an important group of plant ssDNA viruses capable of replicating in the host nuclei using dsDNA intermediates via the rolling circle mechanism. Members of the family *Geminiviridae* have circular, ssDNA genomes encapsidated in twinned icosahedral particles [1, 2]. Geminiviruses are divided into four genera, *Mastrevirus*, *Curtovirus*, *Topocuvirus* and *Begomovirus*, based on the viral genome structure, host range and type of insect vector [3, 4]. Members of the genus *Begomovirus* are either monopartite (one ~2.9-kb DNA) or bipartite (two ~2.7-kb DNAs referred to as ''DNA A'' and ''DNA B''). They are transmitted by whiteflies and infect dicotyledonous plants. *Mungbean yellow mosaic virus* (MYMV) is a bipartite begomovirus causing severe yellow mosaic disease of mungbean (*Vigna radiata*) in Thailand [5] and blackgram (*V. mungo*) in Southern India [6].

Pathogen-derived resistance (PDR) is a very effective genetic engineering approach to control plant viruses [reviewed in 7]. An important pre-requisite for the use of PDR is to ensure that the pathogen-derived

gene does not interfere with essential host functions. Geminiviruses encode specific genes essential for replication, encapsidation of their genome, cell-to-cell movement, systemic movement and insect transmission. Many of these genes are excellent candidates for developing host resistance based on PDR. Coat protein (CP) expression strategy against *Tomato yellow leaf curl virus* (TYLCV) [8] resulted in resistance in tomato lines with CP expression. Expression of truncated CP of *Tomato mottle virus* (deletion of N-terminal 30 amino acids) also conferred resistance [9]. Recently, Raj et al. [10] reported resistance of transgenic tomato plants harbouring *Tomato leaf curl virus* (TLCV) full-length CP gene in the T_1 generation upon challenging with whiteflies carrying TLCV.

Replication-associated protein (Rep) is an important protein analyzed for PDR in geminiviruses. Rep is a multifunctional protein involved in viral replication, regulation of its own transcription and activation and recruitment of host-encoded proteins related to host DNA synthesis [reviewed in 1]. Rep is the only viral protein that is absolutely essential for viral replication [11]. Its N-terminal domain mediates initiation of DNA replication and transcriptional repression [12, 13] and the nicking and joining activity required for initiation and termination of viral-sense strand DNA synthesis [14]. The central portion of Rep has a role in oligomerization [14], while the C-terminal region contains a nucleoside triphosphate (NTP)-binding domain required for viral replication [15, 16]. Expression of Rep in sense orientation inhibited *African cassava mosaic virus* (ACMV) replication [17]. Chellappan et al. [18] reported the production of transgenic cassava plants with full-length and modified Rep with significant broad-spectrum resistance. Hanson and Maxwell [19] studied the effects of transgenic Rep genes (wild type and mutant Rep) from Bean golden mosaic virus-Guatemala (BGMV-GA) on the replication of several bipartite geminiviruses in the tobacco suspension cell culture system. They found that DNA nicking-domain mutant and NTP-binding domain mutant interfered in the replication of BGMV-GA as well as BGMV-Brazil, BGMV-Dominican Republic and, *Bean yellow dwarf virus*. A mutation of NTP-binding domain of Rep caused inhibition of ACMV replication in *Nicotiana benthamiana* [20]. Rep sequence in antisense orientation was used to develop resistance against *Tomato golden mosaic virus* (TGMV) [21], *Beet curly top virus* (BCTV) [22], *Tomato yellow leaf curl Sardinia virus* [23] and *Bean golden mosaic virus*-(Bz) [24]. Interestingly, expression of truncated Rep (T-Rep; N-terminal 210 aminoacids with the deletion of C-terminal NTP-binding site) of TYLCV was very effective in an artificial host [25], as well as in its natural host, tomato [26]. The expression of T-Rep inhibited viral-sense strand synthesis and repressed its own transcription [27]. In the case of homologous viruses, T-Rep-mediated resistance is through repression of Rep gene transcription. If the virus is heterologous, then the interference is due to Rep–Rep oligomerization [28]. A truncated Rep of TYLCV-Israel [mild] expressing the N-terminal 129 amino acids resulted in resistance against TYLCV-Is [mild] [29]. The expression of the oligomerization domain of *Tomato yellow leaf curl New Delhi virus* Rep interfered in DNA accumulation of not only the homologous virus, but also of ACMV, *Pepper huasteco virus* and *Potato yellow mosaic virus* [30].

DNA B-encoded movement proteins, NSP (nuclear shuttle protein, BV1) and MP (movement protein, BC1) are responsible for cooperative movement of viral DNA within and between cells. Von Arnim and Stanley [31] developed resistance against ACMV by expressing its MP. A mutated MP expressed in transgenic tobacco plants, delayed symptom development of ToMoV-(F1) [32]. Delayed symptoms of ToMoV were observed when tomato plants were transformed with NSP or MP genes of *Bean dwarf mosaic virus* [33].

MYMV infects five important pulse crops, blackgram, mungbean, Frenchbean, pigeonpea and soybean, causing an annual yield loss of about $300 million [34]. If the plants are infected at the seedling stage, it can lead to 85–100% yield loss [35]. The application of PDR strategy against MYMV in blackgram and mungbean is rendered difficult due to recalcitrance of these pulses to *Agrobacterium*-mediated transformation. Therefore, an attempt was made to develop tobacco as a model plant to study PDR in transgenic plants harbouring MYMV genes. MYMV inoculation of tobacco (*Nicotiana tabacum*) did not lead to the development of viral symptoms [36]. Here, we report that tobacco leaf discs agroinoculated with partial dimers of MYMV-Vig support viral replication. We developed transgenic tobacco plants with CP, Rep-sense, Rep-antisense, truncated Rep (T-Rep), nuclear shuttle protein (NSP) and movement protein (MP) genes. Leaf discs from these transgenic plants were challenged by agroinoculation with partial dimers of MYMV-Vig [37, 38]. The accumulation of MYMV DNA was higher in the leaf discs of transgenic tobacco plants harbouring CP and MP genes. However, two transgenic plants expressing T-Rep and one expressing Rep-sense, consistently showed inhibition of MYMV-Vig replication. We demonstrate that the simple leaf disc assay in a non-host model plant (tobacco) can be used to evaluate PDR in transgenic plants harbouring MYMV genes.

Materials and methods

Viral clones

Accession numbers of DNA A and DNA B (KA22) of MYMV-Vig in EMBL/Genbank are AJ132575 and AJ132574, respectively. Both DNAs were cloned from field-infected *Vigna mungo* (blackgram) plants [6].

Chemicals, enzymes and kits

Most of the chemicals were purchased from Himedia Laboratories Pvt. Ltd. India and Qualigens Fine Chemicals, India. For tobacco transformation, kanamycin (Kancin; Alembic Chemical Works Co. India) and cefotaxime (Omnatax; Nicholas Piramal India Ltd., Mumbai, India) were purchased from pharmaceutical stores. Hygromycin was purchased from Calbiochem, San Diego, USA. Fine chemicals and antibiotics for bacterial work were purchased from Sigma Chemical Co., St. Louis, USA. Restriction enzymes and Megaprime^{TM} labelling systems were purchased from Amersham Pharmacia Biotech (Little Chalfont, UK) and Roche Applied Science (Mannheim, Germany). Oligonucleotides were bought from Microsynth AG, Switzerland. Nylon membrane (Zetaprobe; Bio-Rad Laboratories, California, USA) was used for Southern hybridization analysis. Nylon N^+ membrane (Roche Applied Science) was used for RNA analysis. [α-^{32}P]dCTP was obtained from Board of Radiation and Isotope Technology, Govt. of India.

Plasmid construction and introduction into *Agrobacterium*

Rep-sense and Rep-antisense binary plasmids were constructed as follows. A 1.28-kb PstI/SspI fragment encoding the Rep of MYMV-Vig (DNA A nucleotide positions 2722 to 1446) was subcloned into pIC19H in *Pst*I/*Eco*RV sites. From this clone, a 1.3-kb *Hin*dIII fragment having Rep ORF was cloned downstream of CaMV 35S promoter in the binary vector pGA643 [39], with *npt*II as plant selection marker. The plasmid pGA-Rep has Rep in sense orientation (Fig. 1A). A truncated version of Rep (DNA A nucleotide positions 2613 to 1973) was constructed as follows: A mutagenic PCR was carried out using *Pfu* polymerase with oligonucleotides, 5′-tatttttctagagaaaatatgcctagactcggtcg-3′ (embedding a *Xba*I site, underlined) and 5′-taagaattcttt acggcgcgcagctatctcttac-3′ (embedding an *Eco*RI site, underlined) to amplify a 648-bp fragment (cycling parameters-95°C for 5 min and then 30 cycles of 95°C for 40 s, 58°C for 40 s, 72°C for 1 min, and a final extension of 72°C for 5 min) which encodes the N-terminal 210 amino acids and a deletion of C-terminal 142 amino acids. A partial dimer clone of MYMV-Vig DNA A (pMT2 in pBSIIKS$^+$ backbone; lab collection) was used as a template. This PCR product was purified (QIAGEN, Valencia, CA, USA), digested with *Eco*RI and *Xba*I and cloned into the pJIC35S cassette [40] between CaMV 35S promoter and terminator. Both strands of the cloned fragment were sequenced using ABI PRISM 310 automated sequencer. An *Eco*RV fragment having the T-Rep gene with 35S promoter and terminator was then subcloned in the *Sma*I site of pCAMBIA2301 (pCam-T-Rep; 12.9-kb) (Fig. 1B). A binary plasmid with CP ORF (DNA A nucleotide positions 147 to 1102) was constructed as follows: pOK-CP is a plasmid having the 0.9-kb of CP ORF as a *Bam*HI/*Hin*dIII fragment. From this plasmid, CP ORF was subcloned into pJIC35S cassette. An *Eco*RV fragment with an expression cassette from pJIC35S having CP was then placed into the *Sma*I site of pCAMBIA1301 (pCam-CP 13.5-kb, hygromycin as plant selection marker) (Fig. 1C).

A binary vector with MP ORF (DNA B nucleotide positions 2117 to 1221) of MYMV-Vig DNA B KA22 was constructed as follows: A mutagenic PCR to amplify a 896-bp fragment containing MP ORF was carried out using oligonucleotides 5′-acacactcgaggaataatggagaatt-attcagg-3′ (embedding a *Xho*I site, underlined) and 5′-ataaagcatgcgtgttacaacgctttgttcac-3′ (embedding a *Sph*I site, underlined). The cycling parameters were 95°C for 5 min and then 30 cycles of 95°C for 40 s, 59°C for 40 s, 72°C for 1 min and a final extension of 72°C for 5 min. The PCR product was purified, digested with *Xho*I and *Sph*I and subcloned into pOK12 in the corresponding sites. After verifying the sequence of the resultant clone, a *Spe*I/*Sac*I fragment having the MP ORF was subcloned into pJIC35S cassette between CaMV 35S promoter and terminator. The expression cassette was subcloned as an *Eco*RV fragment (1.6-kb) into the binary vector pCAMBIA2300 (pCam-MP-22; 10.3-kb) (Fig. 1D).

The binary plasmids were mobilized from *E. coli* into *Agrobacterium tumefaciens* by triparental mating. All mobilizations were confirmed by Southern hybridization analysis.

Tobacco transformation

Tobacco (*N. tabacum* L. cv. Wisconsin 38) leaf discs (8-mm diameter) were cut using a cork borer from 3- to 4-week-old, axenically grown plants. Transformation of leaf discs was performed as described earlier [41]. Transgenic shoots were rooted in BGS medium (MS salts, 0.001% folic acid, 100 mg/l myoinositol, 0.4 mg/l

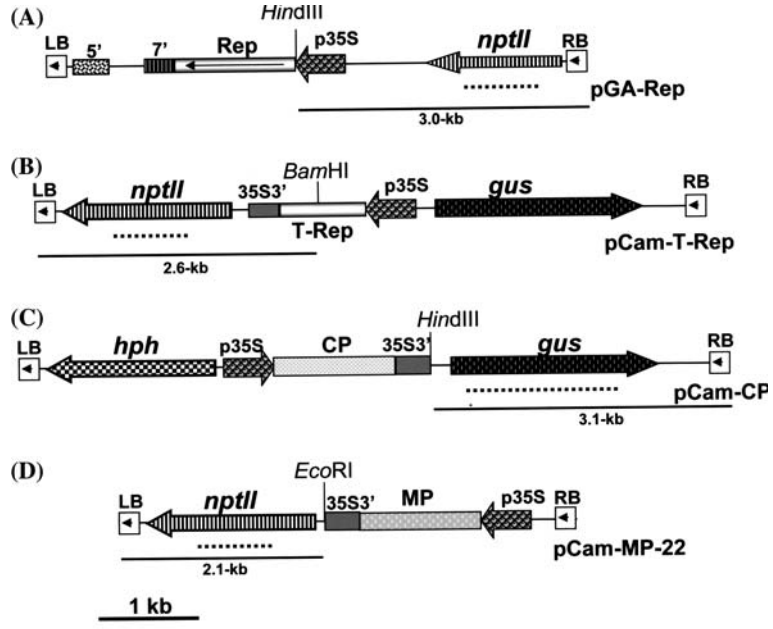

Fig. 1 Binary plasmids with MYMV-Vig genes used for the transformation of tobacco plants. Only T-DNA regions are represented. (**A**) Plasmid pGA-Rep harbours MYMV-Vig Rep gene in sense orientation under CaMV 35S promoter in pGA643 [39]. It has *npt*II as plant selection marker. (**B**) Binary plasmid pCam-T-Rep harbours truncated Rep (which can express N-terminal 210 aminoacids) under 35S promoter in pCAMBIA2301. This plasmid has *npt*II as plant selection marker and *int-gus* as reporter. (**C**) Binary plasmid pCam-CP, which has CP under 35S promoter in pCAMBIA1301 with *hph* as plant selection marker. (**D**) Binary plasmid pCam-MP-22 is a derivative of pCAMBIA2300 with *npt*II as plant selection marker, harbouring MP gene of MYMV-Vig. RB: T-DNA border-right, p35S: CaMV 35S promoter, 7′: transcription termination of gene 7 of pTiA6, 5′: transcription termination of gene 5 of pTiA6, *npt*II: neomycin phosphotransferase II, *gus*: *β*-glucuronidase, 35S3′: CaMV 35S polyA, *hph*: hygromycin phosphotransferase, LB: T-DNA border-left. Dotted lines indicate the region covered by the probe taken for Southern hybridization analysis. Restriction enzyme sites used for junction fragment analysis are stated. The junction fragment portions between the restriction sites in the T-DNA and the borders are shown as bold lines. The minimum lengths of junction fragments are marked

thiamine, 0.057 μM indole-3-acetic acid, 0.14 μM kinetin, 3% (w/v) sucrose, 0.9% (w/v) agar, pH 5.7) supplemented with 250 mg/l cefotaxime and with either 50 mg/l kanamycin (in all cases except pCam-CP), or 50 mg/l hygromycin (for pCam-CP).

Agroinoculation

Agroinoculation of tobacco leaf discs was performed with *A. tumefaciens* strain Ach5 harbouring partial dimers of both DNA A and DNA B of MYMV-Vig [38]. After 2 days of co-cultivation, tobacco (control and transgenic) leaf discs were transferred to tobacco shoot-induction medium (MS salts, B5 vitamins, 0.5 μM NAA, 4 μM BAP, 3% (w/v) sucrose, 0.9% (w/v) agar, adjusted to pH 5.7) supplemented with 250 mg/l of cefotaxime.

DNA analysis

Total DNA was extracted from control and transgenic tobacco plants and from leaf discs as described by Rogers and Bendich [42]. Plant DNA was estimated using a Hoefer DNA fluorometer (DyNA Quant 200) using Hoechst dye 33258. Southern blot analyses were carried out using total DNA digested for 12 h with appropriate enzymes, separated in 0.8–1% agarose gels in 1× TBE or 1× TNE (40 mM Tris–acetate, pH 7.5, 20 mM sodium acetate, 2 mM EDTA) buffer [17]. S1 nuclease treatment was performed to cleave ssDNA [43] in order to distinguish between ssDNA and dsDNA forms of the virus. Five micrograms of DNA was used for S1 treatment. S1 nuclease at 1.2 units/μg of DNA was added to a reaction mixture containing 1.2 μg of denatured calf thymus DNA and 1× reaction buffer [50 mM sodium acetate (pH 5.6), 280 mM NaCl and 4.5 mM zinc sulphate], and incubated for 30 min at 37°C. The reaction was stopped using 0.1 vol of 0.1 M EDTA (pH 8.0) and used for Southern hybridization analysis. The copy number of viral DNA in tobacco leaf discs was determined by Southern hybridization analysis using copy number reconstructions. Full-length DNA A of MYMV-Vig (2.8-kb) equivalent to

1-copy, 10, 100 and 1,000 copies per one copy of tobacco genome, was mixed with 5 μg of tobacco DNA. For example, 1-copy reconstruct has 3.1 pg of full-length MYMV-Vig DNA A in 5 μg of tobacco DNA. About 5 μg of total DNA from control and agroinoculated tobacco leaf discs was analyzed by Southern blotting with or without S1 treatment.

Total RNA extraction and northern blot analysis

Approximately 1.0 g of young leaf tissue from the transgenic and non-transgenic plants was ground using liquid nitrogen. Total RNA was extracted as described by Pawlowski et al. [44], quantified at 260 nm in a spectrophotometer and used for northern blot analysis. Ten micrograms of total RNA samples from control and transgenic tobacco plants were separated at 100 V in a 1.5% agarose gel containing 1% formaldehyde. Ethidium bromide-stained rRNA bands were used to check equal loading of total RNA in all lanes. Formaldehyde was removed from the gel by elution buffer. RNA was transferred to nylon N^+ membrane by capillary transfer. Northern hybridization analysis was done as described by Pawlowski et al. [44]. A 1.2-kb Rep coding sequence, labelled with [α-^{32}P]dCTP, was used as probe.

Results

A tobacco leaf disc agroinoculation assay to study MYMV-Vig DNA replication

A single-strain of *A. tumefaciens* (Ach5) harbouring both DNA A and DNA B partial dimers in separate binary vectors was used for inoculation of tobacco leaf discs [38]. Five days, 10 and 15 days after agroinoculation, the leaf discs were harvested, DNA extracted and subjected to Southern hybridization analysis. Figure 2A represents the analysis with MYMV-Vig DNA A probe. Viral DNA was present in all three time points (panel A). Highest accumulation of viral DNA [both single-stranded (ss) and double-stranded (ds)] was found in the leaf discs collected 10 days after agroinoculation. An intense band at 2.7-kb, representing the replicative form of the virus, could be seen in the leaf discs harvested after 10 days, following S1 treatment of DNA. Signals of high-molecular weight (10-kb and above) correspond to the binary vectors, which carry the partial dimers of the virus. To check the accumulation of DNA B in the leaf discs incubated for 10 days after agroinoculation, total DNA was analyzed (Fig. 2B) with MYMV-Vig DNA B-specific probe devoid of common region. DNA B accumulation was observed in DNA samples with and without S1 treatment. Since viral DNA accumulation was maximum 10 days after agroinoculation, we used this condition in all virus replication assays.

To find out the efficiency of MYMV DNA replication in tobacco and to estimate the viral DNA copy number, a Southern hybridization experiment was performed with copy number reconstructions. Copy number reconstructions representing 1, 10, 100 and 1,000 copies of MYMV DNA A for each copy of tobacco genome were prepared in 5 μg aliquots of tobacco DNA. These samples were analyzed in a gel along with 5 μg total DNA from control and agroinoculated leaf discs. S1 treatment was done to distinguish ssDNA and dsDNA (Fig. 2C). Following Southern hybridization analysis with DNA A probe, the portions of the nylon membrane corresponding to 1, 10, 100 and 1,000 copies and viral DNA (–S1 lane) were excised and radioactivity was measured in a scintillation counter. The values were used to prepare a standard curve (not shown). From the standard curve, the copy number of MYMV DNA in tobacco leaf discs was estimated as 90 copies per tobacco genome. Thus, MYMV-Vig replication is efficient in tobacco leaf discs.

Accumulation of MYMV-Vig DNA in agroinoculated leaf discs of transgenic plants with MYMV-Vig genes

Tobacco transformation was performed [41] with the *A. tumefaciens* vir helper strains harbouring MYMV-Vig-derived genes. Kanamycin-resistant or hygromycin-resistant shoots were sub cultured in the tobacco shoot-induction medium at intervals of 14 days. Southern hybridization analysis was done to confirm T-DNA integration and to determine the T-DNA copy number (results not shown). Details of the probes used and the expected sizes of the junction fragments are presented in Fig. 1. Southern hybridization analysis was done to study MYMV-Vig replication and the accumulation of its DNA in agroinoculated tobacco leaf discs. S1 nuclease treatment was used to distinguish between ssDNA and dsDNA intermediates [43]. The three Rep-sense transgenic plants, 8A, 8B and 8C have single-copy insertions of the transgene. Upon agroinoculation of the leaf discs with DNA A and DNA B, viral DNA accumulation was comparable between control and the transgenic plant 8C (Fig. 3A). Contrasting results were obtained between the other two

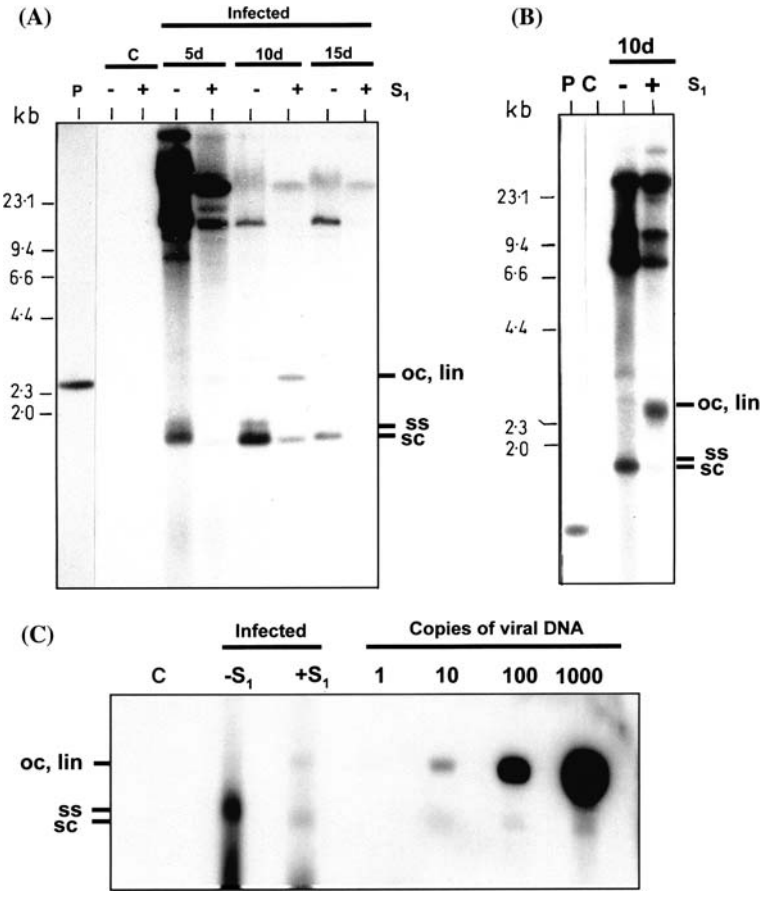

Fig. 2 Southern blot analysis of MYMV-Vig DNA in agroinoculated tobacco leaf discs. The blots were hybridized with DNA A probe (panel **A** and **C**) or DNA B-specific probe (panel **B**). Leaf discs were agroinoculated with *A. tumefaciens* strain Ach5 harbouring partial dimers of DNA A and DNA B of MYMV-Vig [38] Total DNA was extracted from control (lane C) and agroinoculated leaf discs after 5 days (5 d), 10 days (10 d) and 15 days (15 d) after infection. In lane P, 250 pg of probe DNA was loaded as positive control [full-length 2.7-kb DNA A as *Pst*I fragment in panel **A**, a 1.0-kb *Bam*HI/*Cla*I fragment (without CR) from DNA B in panel **B**]. In panel **C**, copy number reconstructions with 1, 10, 100 and 1,000 viral DNA copies of MYMV DNA A per tobacco genome were prepared and loaded along with 5 μg of total DNA from control plant. DNA from control (lane C) and from agroinoculated leaf discs (–S1, +S1) was analyzed. Total DNA (5 μg) was loaded in each lane and electrophoresed in a 0.8% agarose gel in TNE buffer [17], transferred to a nylon membrane (Zeta-probe) and hybridized with [α-^{32}P]dCTP-labelled DNA A or DNA B probe. Open circular (oc), linear (lin), single-stranded (ss) and super-coiled (sc) forms of MYMV-Vig DNA are marked [43]. Positions of *λ*/*Hin*dIII fragments are marked and their sizes in kb are indicated. +: Pretreated with S1 nuclease,–: absence of S1 nuclease

Rep-sense transgenic plants, 8A and 8B. Interestingly, the plant 8A showed unusually high levels of ssDNA and plant 8B had virtually no viral DNA (Fig. 3A). Similar results were obtained when agroinoculation experiments were repeated. A non-denaturation Southern transfer was done for plant 8A to confirm the ssDNA nature of the 1.8-kb band. As expected, only 1.8-kb band (ssDNA) hybridized in non-denatured DNA blot probed with DNA A (Fig. 3A; right panel; non-denatured). The Rep-sense transgenic plant 8B completely inhibited viral DNA accumulation.

The T-Rep transgenic plants T1 and T4 have single-copy insertions and T2 and T5 have two and multiple T-DNA copies, respectively (results not shown). Viral DNA accumulation was comparable in control plant and the T-Rep transgenic plants T2 and T5 (Fig. 3B). However, plants T1 and T4 inhibited the accumulation of viral DNA. The viral titre was many folds lesser in the T-Rep plants T1 and T4. The inhibition of viral DNA accumulation in T-Rep plants T1 and T4 was confirmed by repeating the agroinoculation experiments. Thus, two T-Rep plants, T1 and T4, both of which are single-copy transgenic plants, effectively inhibited viral DNA accumulation.

The leaf disc virus replication assay was performed in six transgenic tobacco plants transformed with CP.

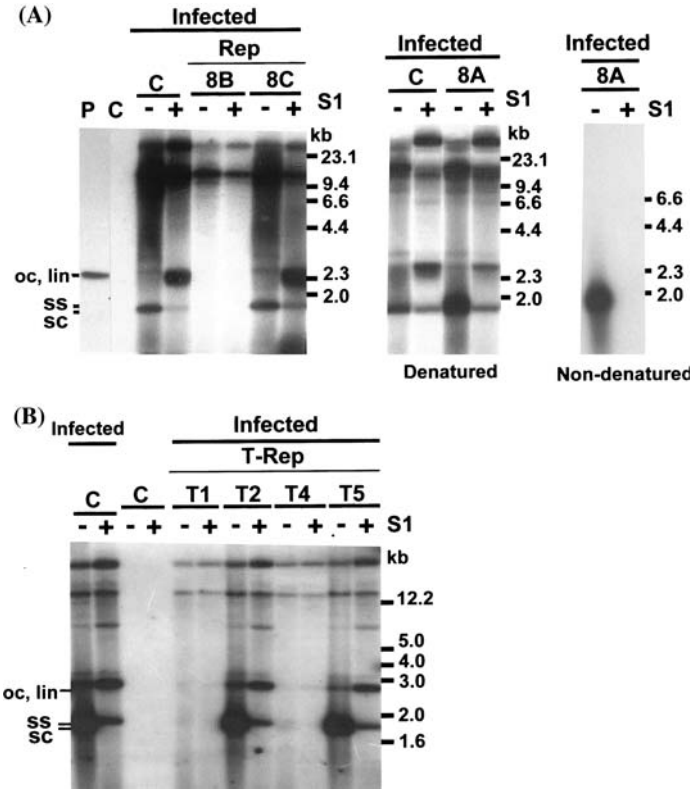

Fig. 3 Southern blot analysis of MYMV-Vig DNA in agroinoculated leaf discs of control and transgenic tobacco plants with Rep-derived sequences. Leaf discs were agroinoculated with *A. tumefaciens* strain Ach5 harbouring partial dimers of MYMV-Vig DNA A and DNA B (Infected). (**A**) Southern analysis of Rep-sense transformed tobacco plants, 8A, 8B and 8C, (**B**) Southern analysis of T-Rep plants, T1, T2, T4 and T5. Control lanes: Probe DNA-250 pg of 2.7-kb *Pst*I fragment of MYMV-Vig DNA A (lane P); total DNA from non-agroinoculated control tobacco leaf discs (lane C); total DNA from agroinoculated control tobacco leaf discs (lane C, Infected). In all the blots, 5 μg of total DNA (extracted 10 days post-agroinoculation) was loaded in each lane and electrophoresed in a 1.0% agarose gel in 1× TNE buffer [17], transferred to a nylon membrane (Zeta-probe) and hybridized with [α-^{32}P]dCTP-labelled DNA A probe. Open circular (oc), linear (lin), single-stranded (ss) and super-coiled (sc) forms of MYMV-Vig DNA are marked. One-kb ladder or λ/*Hin*dIII size markers used are indicated in kb sizes. +: Pretreated with S1 nuclease, –: absence of S1 nuclease. DNA from the agroinoculated plant 8A was subjected to denatured and non-denatured Southern transfer to analyze ssDNA. Denatured: gel treated with alkali before transfer, non-denatured: gel soaked in 20× SSC before transfer (no alkali treatment)

Interestingly, all the transgenic plants showed higher accumulation of viral DNA when compared to the controls (Fig. 4A). In none of the six transgenic plants, viral DNA accumulation was inhibited. Five plants transformed with MP were taken for tobacco leaf disc assay. In the transgenic plants MP2, MP3, MP4 and MP5, the bands corresponding to the positions of ssDNA and dsDNA were more intense. The band corresponding to open circular (oc) and linear (lin) RF exhibited unusual mobility upon S1 treatment (Fig. 4B). In all these lanes, clear smear could be seen below the oc/lin form of the viral DNA. The reason for the unusual mobility is not clear. Overall, three transgenic tobacco plants, two with T-Rep gene and one with Rep-sense gene strongly inhibited MYMV-Vig DNA accumulation in leaf discs.

Inhibition of MYMV-Vig accumulation is associated with strong transgene mRNA expression

Northern hybridization analysis was done to study whether the inhibition of MYMV-Vig replication in the transgenic tobacco plants 8B, T1 and T4 is associated with the expression of the pathogen (MYMV-Vig)-derived genes. Total RNA was separated in a 1.5% formaldehyde-agarose gel and hybridized to the full-length Rep gene probe (Fig. 5). Total RNA from an untransformed tobacco plant served as a negative control (lane C). An intense 1.4-kb RNA band could be seen in plant 8B (Rep-sense) that inhibited viral DNA accumulation. On the other hand, the plant 8C (Rep-sense), which did not inhibit viral accumulation

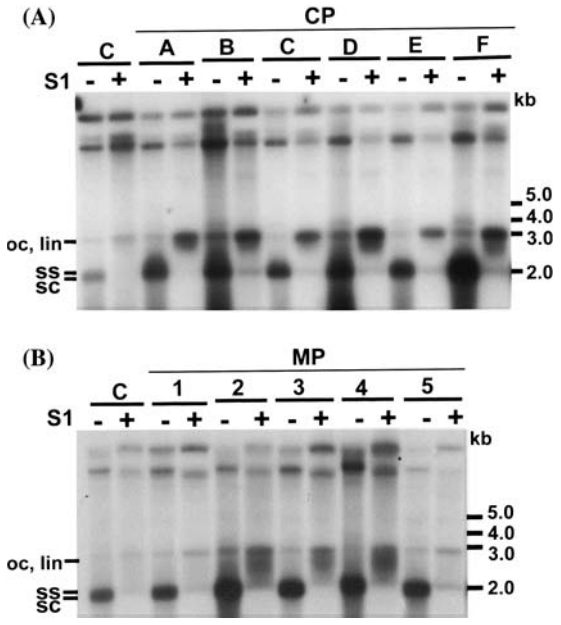

Fig. 4 Southern blot analysis of MYMV-Vig DNA in agroinoculated leaf discs of control and transgenic tobacco plants with CP and MP genes. (**A**) Analysis of CP-transformed tobacco plants, CP-A, CP-B, CP-C, CP-D, CP-E and CP-F. (**B**) Analysis of MP-transformed tobacco plants MP-1, MP-2, MP-3, MP-4, and MP-5. For other details refer to Fig. 3 legend

in leaf disc assay, has very low expression as represented by a very weak 1.4-kb RNA band. RNA extracted from a tobacco plant 9A transformed with MYMV-Vig Rep-antisense gene, was included in this analysis. A 1.4-kb RNA, corresponding to the Rep-antisense RNA, accumulated in this plant (Fig. 5). The

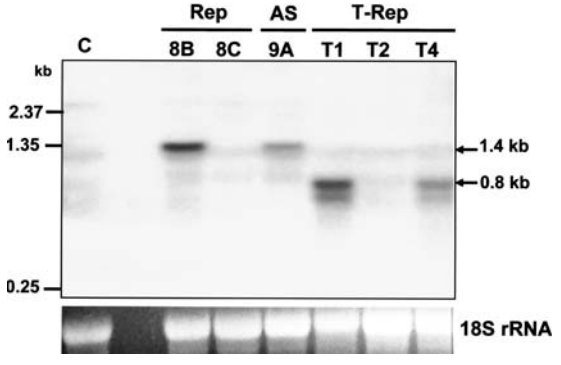

Fig. 5 Northern blot analysis of RNA extracted from control and transgenic tobacco plants. Total RNA (10 μg) from control plants (lane C) and transgenic plants with Rep-sense gene (8B, 8C), Rep-antisense gene (AS/9A) and T-Rep gene (T1, T2 and T4) was separated in a 1.5% agarose gel with 1% formaldehyde and transferred to nylon N^+ membrane. The 18S rRNA portion of the ethidium bromide-stained gel is placed at the bottom to show equal loading of RNA in all the lanes. Sizes of RNA standard (Invitrogen) are marked on the left. The sizes of Rep (1.4-kb) and T-Rep (0.8-kb) RNAs are marked on the right. A 1.3-kb fragment of MYMV-Vig Rep gene was labelled with [α-^{32}P]dCTP and used as probe

T-Rep transgenic plants, T1 and T4, showed high accumulation of the expected a 0.8-kb truncated Rep RNA. These two plants strongly inhibited viral DNA accumulation in the leaf disc assay. Another T-Rep plant T2, that did not inhibit viral DNA accumulation, did not express the 0.8-kb RNA. The results show that inhibition of MYMV-Vig DNA accumulation in the leaf discs of transgenic tobacco plants with Rep-sense and T-Rep genes is associated with the high level expression of the corresponding transgenes.

Discussion

Agroinoculation of leaf discs is a simple and rapid assay to study geminivirus replication [11]. *A. tumefaciens* strains, harbouring partial dimers in the T-DNA of binary vectors, are used for agroinoculation [37]. Following T-DNA-mediated transfer of the partial dimer of a geminivirus into plant cells, the viral component is released to replicate as a circular, double-stranded replicative form [45]. Both ssDNA and dsDNA forms of viral DNA could be detected by Southern blot analysis. Many previous reports clearly showed that the leaf disc assay is a relatively simple technique useful to study the effect of transgenes on viral replication. In this study, the tobacco leaf disc replication assay was effectively used to evaluate the effect of MYMV-Vig-derived genes in transgenic plants on viral DNA accumulation.

Accumulation of viral DNA reached the highest level in the leaf discs collected 10 days after agroinoculation (Fig. 2A). Our results corroborate well with the previous work of Bejarano and Lichtenstein [22], where the authors showed that TGMV DNA A levels in tobacco leaf discs peaked during 7–10 days after agroinoculation. Transgenic *N. benthamiana* leaf discs agroinoculated with ACMV were harvested 9 days after agroinoculation to study the effect of Rep transgene on viral replication [17]. Castillo et al. [46] observed the inhibitory effect of *LeSUMO* (small ubiquitin like modifier from *Lycopersicon esculentum*) on TGMV accumulation in tobacco leaf discs eight days after agroinoculation. Interestingly, it took a much longer time of 16–45 days after agroinoculation to observe the effect of *LeSUMO* on viral replication in the whole plant. Tobacco leaf discs effectively supported MYMV-Vig replication. Both single and double-stranded viral DNA forms accumulated. We estimated the number of copies of MYMV DNA A as 90 per tobacco cell (Fig. 2C).

We generated many morphologically normal-looking transgenic tobacco plants harbouring Rep-sense, T-Rep, CP and MP genes. The T-DNA integration in

these plants was confirmed by Southern hybridization analysis and transgene copy numbers were determined. Hong and Stanley [17] successfully generated transgenic *N. benthamiana* plants harbouring full-length copy of Rep gene. However, Bendahmane and Gronenborn [23] were unable to generate *N. benthamiana* plants that expressed the sense copy of the complete Rep gene of TYLCV. This observation was attributed to the possible deleterious effect of the constitutively expressed active Rep protein. In our experiments, all the Rep gene-transformed tobacco plants were phenotypically normal. Many transgenic plants expressing the geminivirus NSP and MP genes were reported to be phenotypically abnormal [31, 33]. However in our experiments, transgenic plants with the MP gene were phenotypically normal.

In one transgenic plant with Rep-sense (8B) gene and two transgenic plants with T-Rep (T1 and T4) accumulation of MYMV-Vig DNA was reduced (Fig. 3). Interestingly, in all the three tobacco plants, 8B, T1 and T4 which exhibited a reduction in viral DNA accumulation, expression of the RNA of the corresponding MYMV-Vig genes was high (Fig. 5). In the plants 8C (Rep-sense) and T2 (T-Rep), in which MYMV-Vig replication was not inhibited, the corresponding RNA signals were low or absent. Three Rep-antisense transgenic tobacco plants did not cause any reduction in MYMV-Vig DNA accumulation (data not shown). The Rep-antisense transgenic plant 9A, which did not inhibit viral replication, expressed the Rep-antisense RNA at a lower level (Fig. 5). The failure of 9A (Rep-antisense) to inhibit viral replication and marked inhibition of virus replication in the plants 8B (Rep-sense), T1 and T4 (T-Rep) suggest that synthesis of sense RNA and the possible accumulation of Rep protein may have a direct role in interfering with the accumulation of the viral DNA. The observations that 8B, T1 and T4 are single-copy transgenic plants and they accumulated high levels of Rep mRNA, rule out the role of RNA silencing in the observed inhibition of MYMV replication. Since the levels of the Rep protein were not determined in these transgenic plants, it is not possible to comment on the role of Rep and truncated Rep proteins in the observed inhibition of MYMV-Vig accumulation. One Rep-expressing plant (8A) accumulated higher amounts of viral ssDNA, but northern hybridization analysis could not be completed on 8A since it was lost due to contamination.

As reported earlier for ACMV in *N. benthamiana* [21], we report that inhibition of MYMV-Vig replication/accumulation in transgenic tobacco plants is associated with the expression of the transgenes encoding Rep or T-Rep. As proposed by Hong and Stanley [17] for ACMV Rep-sense *N. benthamiana* plants, unregulated binding and nicking at *ori* and competition for transcription factor binding sites, may contribute to a reduction in viral replication. Besides, the transgene-encoded Rep may negatively regulate viral Rep gene transcription [13]. MYMV-Vig Rep represses its own expression and its mRNA accumulates to very low levels in infected blackgram [47]. Any unregulated high expression of this RNA may bring about crucial changes in viral replication. The inhibition of viral DNA accumulation in T-Rep-expressing plants is explained on the basis of non-productive protein complex formation by the truncated Rep protein [26–28].

Six transgenic lines, transformed with the MYMV-Vig CP gene, accumulated higher levels of the viral DNA in tobacco leaf discs. This can be attributed to the fact that CP can bind to the freshly generated ssDNA, and promote the continuation of the rolling circle replication [48]. Qin et al. [48] identified a region of CP, which is important for ssDNA binding and for the accumulation of viral DNA. In four of the five transgenic plants harbouring the MP gene, agroinoculation resulted in the accumulation of higher levels of MYMV DNA. The significance of this is not clear. Six transgenic tobacco plants generated with MYMV-Vig NSP gene did not show any change in MYMV-Vig DNA accumulation (data not shown).

Blackgram is highly recalcitrant to *Agrobacterium*-mediated transformation. Though there is one report on generation of transgenic blackgram [49], the conditions are very difficult to reproduce. Thus, it would be useful to deploy an experimental host like *N. tabacum*, to evaluate the benefit of virus-derived genes for inhibiting virus replication in transgenic plants. In the case of TYLCV, a model host *N. benthamiana* was initially used to demonstrate resistance-using T-Rep [25], which was effectively carried forward to the natural host plant, tomato [26]. In a similar strategy, we evaluated the major MYMV-Vig genes that include CP, Rep (sense, antisense and truncated), NSP and MP for PDR by initially raising transgenic tobacco plants. Transformation with the gene AC2, encoding the transcriptional activator protein (TrAP) was not attempted because it has the risk of enhancing susceptibility to viral infections [50]. We show that the leaf disc assay in tobacco, a non-host plant for MYMV-Vig, offers a useful system to study the effect of viral genes in transgenic plants on viral replication. On the basis of the virus replication assays in transgenic tobacco leaf discs, Rep-sense and T-Rep genes of MYMV-Vig emerge as potential candidates to inhibit MYMV replication. These candidate genes hold promise to develop yellow mosaic virus resistance in blackgram.

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