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Screening of Transgenic Tobacco for Resistance Against *Cucumber mosaic virus*

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Abstract: Coat protein (*CP*), Movement Protein (*MP*) and Overlapping (*OVG*) genes were isolated from a Malaysian *Cucumber mosaic virus* (CMV) isolate via RT-PCR and transformed into *Nicotiana tabacum* through *Agrobacterium tumefaciens*-mediated transformation. Out of the thirty six independently transformed lines developed from the three different genes and the mutants of *MP* and *OVG*, five lines were tested for resistance against CMV by challenge inoculations using three different concentrations (1:10, 3:10 and 5:10) of CMV macerated in 0.1 M sodium phosphate buffer (pH 7.0). The transgenic lines exhibiting complete resistance remained symptomless even when re-inoculated with 1:10 concentration of virus. The level of viral RNA accumulation in inoculated leaves was significantly (at least 2-3 times) lower compared to the control untransformed plants. The upper leaves which were analysed for systemic spread of the infection had much lower levels of viral RNA accumulation compared to the inoculated leaves. Amongst the three genes and two mutant lines that were generated in this study, we found that the *CP* and *MP* genes were able to provide a better level of resistance to the plants compared to the overlapping gene.

Key words: *Nicotiana tabacum,* movement protein, coat protein, overlapping gene, disease resistance, transgenic lines, CMV

INTRODUCTION

Cucumber mosaic virus (CMV) has a tripartite single stranded RNA plus-sense genome that contains five genes encoded by three genomic RNAs (Canto *et al*., 1997; Palukaitis and García-Arenal, 2003). The RNAs 1 and 2 (3.4 and 3.0 kb) encodes the proteins la and 2a (molecular weight of 111 and 97 kDa, respectively), which are both components of viral replicase (Hayes and Buck, 1990). An additional protein of unknown function, 2b (11.3 kDa), is encoded by RNA2 from a small Open Reading Frame (ORF) that overlaps the C-terminus of the 2a protein. It is expressed from a subgenomic RNA designated RNA4A (Ding *et al*., 1994, 1995). RNA3 encodes two proteins; the 3a (30 kDa) protein that is involved in viral movement is encoded by the 5'-proximal ORF of RNA3, while the ORF of the coat protein is located in the 3'-proximal half of RNA3. The 24.5 kDa coat protein is translated from a subgenomic RNA4 that is derived from RNA3.

Cucumber mosaic virus has a large host range and infects approximately 800 species of plants in 365 genera of 85 families (Rizzo and Palukaitis, 1988). Other tripartite plant viruses generally have much narrower host range than CMV (Fulton, 1981). Many CMV strains differ in their ability to infect some host plants. The infection and symptom expression of CMV in a host plant is a complex interaction between the genetic materials of the virus and the host genome (Palukaitis and García-Arenal, 2003).

Since, CMV is a causative agent that causes damage and loss of some agronomically important crops, it is therefore essential for us to develop disease resistant varieties to reduce crop losses due to host susceptibility. In viral infections, several genes have been used to generate resistance in plants. Some of these genes are the coat protein, movement protein, replicase and proteases. Here, we have picked two of these genes, i.e., the coat and movement protein genes and another gene that is present in CMV, which is the overlapping gene to generate transformation constructs. Since, the overlapping gene has been reported in CMV, we believe it has an important role in the development of the disease symptoms in plants and therefore may contribute towards the establishment of disease resistance (Ding *et al*., 1995).

The objective of this study was to produce wild type and mutant cDNA clones of coat protein, movement protein and overlapping protein of CMV. These constructs were then transformed into the tobacco plant systems to generate transgenic lines carrying the three different disease resistance genes and their respective mutants. These transgenic lines were then tested for their efficacy in showing a resistant reaction to CMV by

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evaluating disease severity levels for each line. Here, we report the results from the screening of the transgenic lines.

MATERIALS AND METHODS

This research was conducted from November 2004 to November 2008. All research activities were conducted at the School of Bioscience and Biotechnology, Faculty of Science and Technology, Universiti Kebangsaan Malaysia. The glasshouse facilities and controlled environment studies were conducted in the Plant Biotechnology Laboratory in the Institute System Biology, Universiti Kebangsaan Malaysia.

Construct preparation and transformation of *Nicotiana tabacum*

Preparation of CMV-CP construct: The primer was designed using the CP sequence obtained from the GeneBank (http://www.ncbi.nlm.gov). This sequence was then fed into the Premier Primer 5 programme to design appropriate primer sets. Out of the various sets of primers suggested by the system, a suitable pair was selected for use in PCR. RNA from infected plants was isolated as described by McDonald *et al*. (1987). The CMV-CP gene of our CMV isolate was amplified using RNA that was isolated from infected tobacco plants with specific primers P1: 5'-GTGGAGCACGACACACTTGTCTAC-3' and P2: 5'-CGGACTGTCACCC ACACGGTAG-3'.

A ~760 bp gene fragment was amplified with restriction enzyme sites for *Xba*I and *Hind*III incorporated into the forward primer and a *Bam*HI site that was incorporated into the reverse primer. This PCR product was digested with *Xba*I and *Bam*HI restriction enzymes and then ligated into the *Xba*I/ *Bam*HI site of pBluescript KS-(Strategene, USA).

The PCR was conducted using a Robocycler gradient 96 (Strategene, USA). Plasmid DNA (20 ng) was mixed with PCR buffer (10 mM Tris-HCl (pH 8.8), 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100, GibcoBRL), 5 mM dNTPs (Sigma, USA), 100 pM primers (Sigma, USA) and one unit of Deep Vent *Taq* polymerase (BioLAB, USA). The PCR reaction was conducted under conditions of predenaturation at 95°C for 5 min for 1 cycle and 30 cycles for denaturation at 95°C for 1 min, annealing at 55°C for 1 min 30 sec and elongation at 72°C for 1 min. The PCR product was digested with *Bgl*II and *Nsi*I and then ligated to the *Bam*HI and *Pst*I sites of pBluescript KS- to obtain the construct CaMV35S/CMV-CP/Tnos/P35S/ GUS/Tnos.

Preparation of CMV-OVG constructs: The overlapping gene (OVG) was amplified via RT-PCR using specific primers SD6F (5'-ACG GAT CCT GGT CTC CTT ATG GAG AAC CTG TGG-3') and SD7R (5'-AGG ATC CAT GGA TGT GTT GAC AGT AGT GG-3'). The primers and the RNA were obtained as mentioned above. The PCR product was digested with *Bam*HI and ligated into the *BamH*I site of pPCR-Script. The product was purified, sequenced and analyzed using BLAST (Basic Local Alignment Search Tool; NCBI; http://www.ncbi.nlm.gov/). Mutants of OVG were obtained by random mutagenesis using GeneMorph® Random Mutagenesis (Stratagene, USA). The mutation to the overlapping gene was generated using both the Fovg1-5'-CCGGGTCGACCGTCGAACTAGAGTTAGGC-3' and Rovg1-5'-CCCTGCCTCCTCTGTGAATCTAGACTTG-3' and mpA 5'GCC CTG AAG TCA TTA A*AT GCA T*GG C-3' and mpC - 5'GGA T*GC GGG C*TG ATA AAG CTA TT-3'. The methodology for random mutagenesis was as described by the manufacter's protocol.

Preparation of CMV-MP constructs: The *MP* gene was amplified via RT-PCR with a specific primer pair (Fmp1- 5'-GAT TAA GCT TGC ATG GCT TTC CAA GGT ACC AG-3' and Rmp1-5'-CGT CGA CGC TAA AGA CCG TTA ACC ACA TGC-3') digested and ligated into the *BamH*I site of pPCR-Script. The two sites chosen for mutagenesis are codon-70 and codon-90. These two sites are located in a highly conserved region of the *MP* gene and codon-90 has been shown to play an important role in the assimilation and movement of protein from cell to cell (Boccard and Baulcombe, 1993). Mutants for *MP* gene were generated via site-directed mutagenesis through the use of the Site-Directed Mutagenesis Kit (Promega, USA). The site-directed mutagenesis process is conducted by designing a site-directed mutant primer set which is then partnered with a mutation primer included in the Kit. The methodology was as provided by the manufacturer's protocol.

Transformation of construct into *Agrobacterium tumefaciens* The CMV-CP, CMV- OVG and CMV-MP coding sequence were cloned into either pBI121 (for CMV-CP) or pCAMBIA3301 vector. Both pBI121 and pCAMBIA3301 carry CaMV35S promoters and have reporter genes as well as selection genes that are useful in the screening process. The pBI121 has the Kan^R gene for selection on media and the *GUS* gene that can be used in reporting the transcription levels and location of expression in the plant. pCAMBIA has two reporter genes, *uid*A and *Bar* and a Kan^R that facilitates the selection of positively transformed products. Constructs were transformed into DH5á competent cells using the heat shock method (Ausubel *et al*., 1987). Screening of

transformants was done by antibiotic selection, gel electrophoresis and sequencing. The sequencing process was conducted by a commercial sequencing facility (Research Biolab, Malaysia). Positive constructs were subsequently transformed into *Agrobacterium* LBA4404 pAL4404. Young tobacco leaves were aseptically cut at the proximal and distal ends and transferred to MS plates supplemented with 0.5 mg L^{-1} 2,4-D and incubated at 24°C under 16/ 8 h light/dark regime for 3 to 4 days before inoculation with the transformed *A. tumefaciens*.

Polymerase chain reaction, Southern and Northern Blot analysis for transgene in T0 plants: The total genomic DNA was extracted from 1 g leaf tissue of *N. tabacum* as described by Dellaporta *et al*. (1983). To verify the presence of the gene in T0 plants, Polymerase Chain Reaction (PCR) was carried out using the total DNA isolated from each plant and the CP/ OVG/MP specific primers sets of CMV as stated above. The PCR products were electrophoresed with DNA standard in 1% agarose gel. The PCR product size was compared against the molecular marker to determine if the product size matched that of what was predicted via the Premier Primer 5. Integration and copy number of the constructs in the T0 plants were confirmed by Southern blotting where plant genomic DNA (15 µg) was digested with *Hin*dIII, electrophoresed and transferred to nylon membranes as described in Sambrook *et al*. (1989). The blotted fragments were hybridized with a probe prepared from the entire amplified product of CMV-CP, CMV-OVG and the CMV-*MP* genes to determine the exact copy number of each gene inserted into the genome. To determine the transcription of the *CP/OVG/MP* gene introduced into the *N. tabacum* plants, total RNA was extracted from 1 g leaf tissue of selected transgenic plants following the method of Ding *et al*. (1995). The RNA was assayed for the presence of the transgene transcript by using a DNA probe for the CMV-CP, CMV-OVG and CMV-MP genes. The DNA or RNA to be transferred was electrophoresed on 1% agarose gels according to standard procedures described in Sambrook *et al*. (1989) and blotted on a nylon membrane. The probes used for hybridization were prepared according to the random primer labelling method (Fienberg and Vogelstein, 1983). Prehybrization and hybridization were carried out at 42°C with 50% formamide and the blots were washed as instructed by the manufacturer.

Challenge inoculations of transgenic plants for virus resistance: Transgenic plants at 4-6-leaf stage were challenged with 1: 10, 3:10 and 5:10 inoculum (crude extract) prepared from CMV-infected tobacco leaves macerated in 0.1 M sodium phosphate buffer, pH 7.0 containing 1% sodium sulphite (1 mL buffer/100 mg tissue). Inoculated plants were observed daily for 20 days for the development of symptoms and compared with the control (untransformed challenged). Plants that did not develop any symptoms were tested by back inoculation to detect latent infection, if any for another 7 dpi. Table 1 provides the details of inoculation and experimental groups.

The observation of symptoms was conducted on 10 plantlets that were segregated to each test group and test concentration as indicated in Table 1. The disease severity and symptoms were scored as in Table 2.

Table 1: Concentrations used in test groups

	CMV concentration (10 mg mL^{-1})		
	Dilution[a]		
Plantlets	1:10	3:10	5:10
Transgenic CP	10	10	10
Transgenic MP	10	10	10
Transgenic OVG	10	10	10
Transgenic Mutant MP	10	10	10
Transgenic Mutant OVG	10	10	10
Non transgenic	10	10	10

[a]1:10 = 0.91 mg mL^{-1}, 3:10 = 2.31 mg mL^{-1}, 5:10 = 3.33 mg mL^{-1}. Original test were done on 10 samples per dilution, repeats were conducted at 5 samples per test group

Table 2: Disease severity and reaction classes in tobacco plants inoculated with CMV

Disease severity[a]	Reaction class	Level of resistance	Symptoms
None	1	Resistant	No symptoms observed
Moderate	2	Intermediate (moderate)	Weak symptoms, little spot formations
Low	3	Intermediate (low)	Mild spot formation slight crinkly leaves formation, mild mosaic
High	4	Susceptible	Mosaic, crinkly leaves and mild spotting
Very high	5	Very susceptible	High level mosaic, obvious spots, extensive crinkling of leaves, wilting and death

[a]Based on visual inspection of disease symptoms, Note: The reaction classes were determined looking at the disease severity based on the symptoms exhibited by the plants. Observable symptoms provided

RESULTS AND DISCUSSIONS

Chimeric construct and regeneration of transgenic plants: The prepared construct (pCPBI) contained the CMV-CP gene located between the T-DNA borders of the binary plasmid pBI121 (Fig. 1). The construct in *E. coli* (pCPBI) when digested with *Bam*HI revealed the presence of a ~760 bp CMV-CP gene fragment. The gene was then transformed into *Agrobacterium* to create the conjugant (PCPBI2). Transformation of *N. tabacum* resulted in direct shoot initiation from a large number of leaf explants (about 89%) after 4 weeks. Out of the sixty eight putatively transformed shoots, thirty independent shoots were obtained and eleven lines (CP1-CP11), each representing an individual transformation event, were generated.

The RT-PCR method was used to amplify an approximate ~ 0.20 kb product using the primer sets for the overlapping gene (*OVG*). The product was purified, sequenced and analyzed using BLAST (Basic Local Alignment Search Tool; NCBI; http://www.ncbi.nlm.gov). Results of the sequence analysis showed a high level of identity, 100%, 2e-100, score of 400 bits with the nucleotide sequence of *OVG* gene RNA4A and RNA 2 of CMV strain Q (NCBI accession code: X00985 GI59043 and Z21863 GI18139855 respectively). BLAST2seq (http://www.ncbi.nlm.gov) showed that the sequence was 100% identical to the sequence reported by Ding *et al*., (1994) for the CMV overlapping gene (CAA25494 GI59044).

cDNA of *OVG* gene was cloned into pPCR-Script for easier manipulation in cloning and mutagenesis process as this vector carries *AmpR* gene which makes screening easier for putative mutants. Mutants of *OVG* gene were produced using random mutagenesis via the GeneMorph® Random Mutagenesis System (Stratagene, USA). The wild type and mutant (*ovg1, ovg4, ovg5* and *ovg7*) genes were cloned into the *Xba*1/*Bam*HI site pCAMBIA3301 to form contructs: pOVGCAM, povg1CAM, povg4CAM, povg5CAM and povg7CAM (Fig. 2). Explant tissues (74%) were generated from 300 putatively transformed shoots that were obtained. Two hundred and twenty one independent shoots were formed

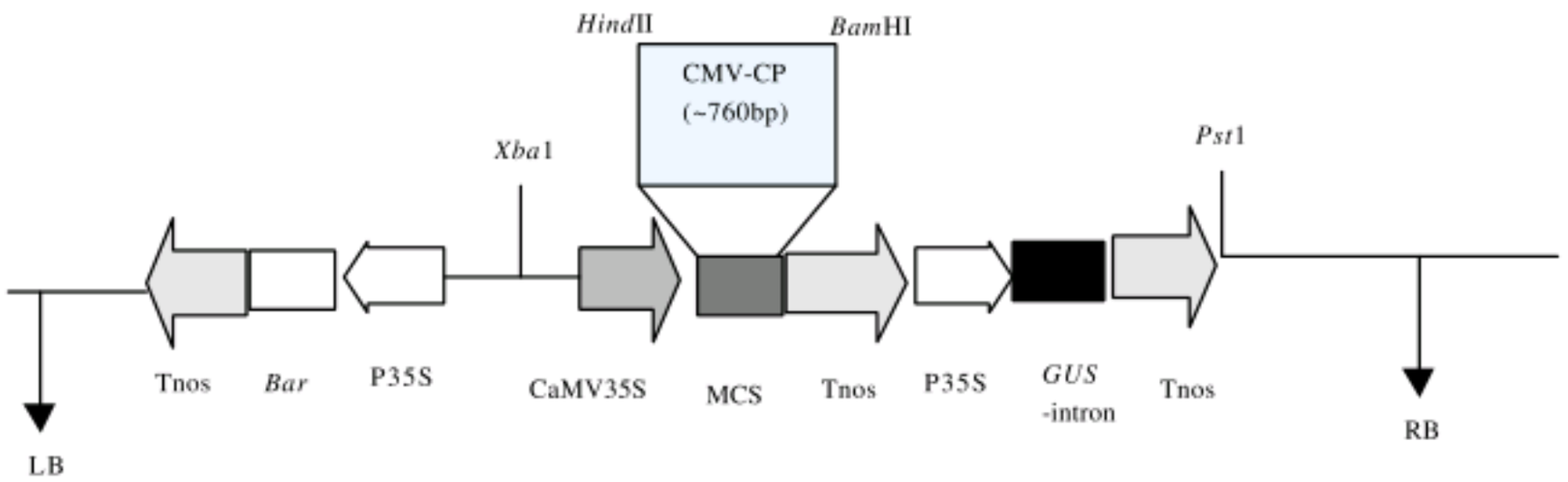

Fig. 1: Linear map of pCPBI2 -TDNA cassette. LB/RB- left/right T-DNA border sequences; P35S/T35S-CaMV 35S promoter/terminator; *bar*-coding region of the phosphinotricin resistance gene; T*nos*-nopaline synthase terminator; gus-intron-*gus*A gene coding region with intron sequence

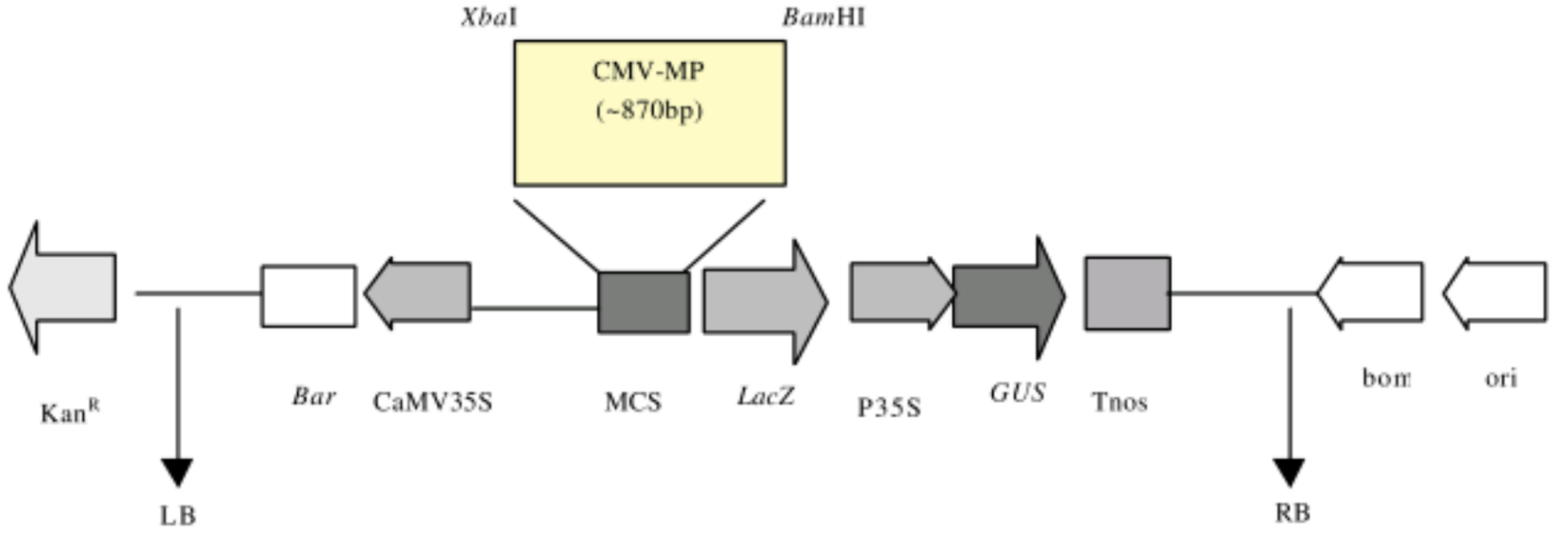

Fig. 2: Linear map of pMPCAM -TDNA cassette. The construct contained the LB/RB- left/right T-DNA border sequences; P35S/T35S-CaMV 35S promoter/terminator; *bar*-coding region of the phosphinotricin resistance gene; Kan^R coding region for kanamycin resistance gene; T*nos*- nopaline synthase terminator; gus-intron-*gus*A gene coding region with intron sequence and the pBR322 bom and pBR322 ori site. The mutations in the *MP* gene were at codon 70 and 90 and their constructs were designated pMPACAM and pMPCCAM, respectively

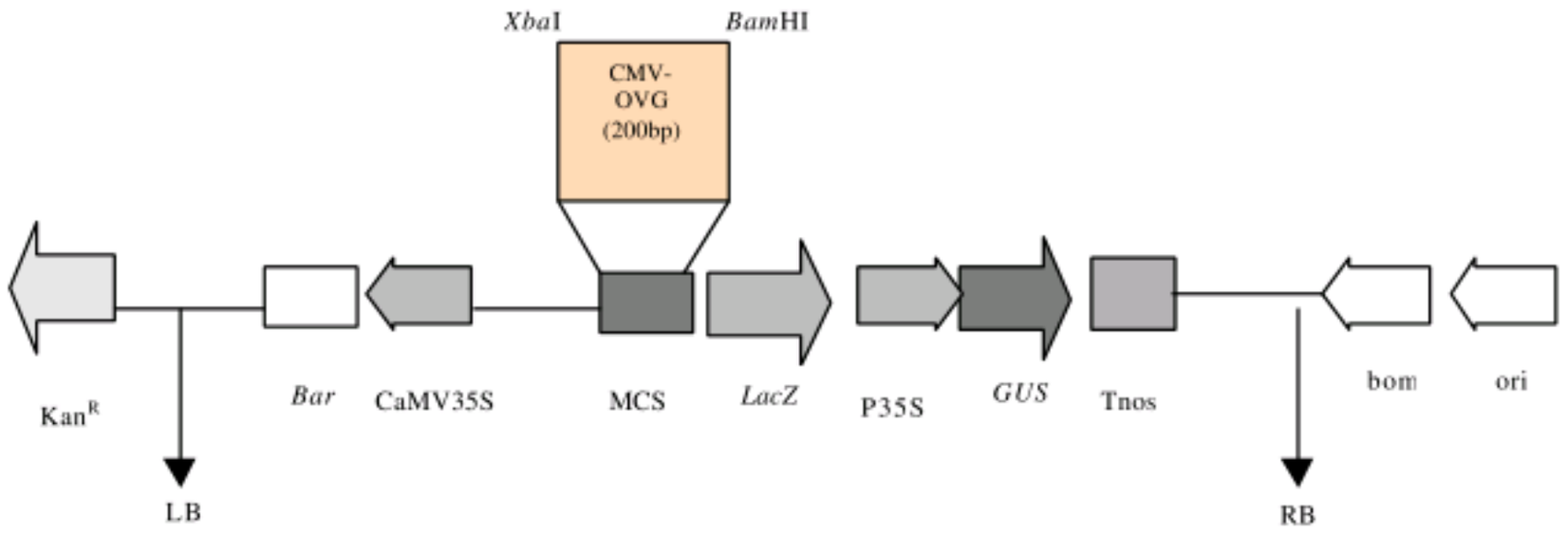

Fig. 3: Linear map of pOVGCAM -TDNA cassette. The construct contained the LB/RB- left/right T-DNA border sequences; P35S/T35S-CaMV 35S promoter/terminator; *bar*-coding region of the phosphinotricin resistance gene; Kan^R coding region for kanamycin resistance gene; T*nos*- nopaline synthase terminator; gus-intron-*gus*A gene coding region with intron sequence and the pBR322 bom and pBR322 ori site. The mutations in the *OVG* gene were randomly generated and these generated the lines used in this study, i.e. povg1CAM, povg4CAM, povg5CAM and povg7CAM

and eleven lines were established and designated OVG1-OVG5 and ovg1, ovg4, ovg4.1, ovg5, ovg7 and ovg7.8; each of which represents independent transformation events.

Similarly cDNA of *MP* gene was cloned into pPCR-Script prior to performing mutagenesis using the Site-directed Mutagenesis System (Promega, USA). The wild type and mutant cDNAs were subcloned into the *Xba*1/*Bam*HI site in pCAMBIA3301 to generate pMPCAM, pMPCCAM and pMPACAM (Fig. 3) and thence into *Agrobacterium* LBA4404 pAL4404. From the explants that were generated, only 56% of the wild type and mutant (mpA and mpC) (5/9 plantlets of each construct were positive) generated were positive. Fourteen lines that were designated MP1-MP4, mpA1-mpA5 and mpC1-mpC5, are independent transformation events produced in this experiment.

All thirty six lines were grown in glasshouse conditions. Acclimatised putative transformed plants grew to maturity and produced normal flowers. However, in several plants, low or no seed setting was observed in certain OVG transformants. Physical deformities in structure of leaves and/or stunted growth was also observed. This we believe is probably due to interference caused by the incorporation of the transgene into the plant genome (Geyer *et al*., 2007; Peretz *et al*., 2007). Every single gene construct transformed generated lines that were sterile. The normal maximum seed setting lines were selected for further use.

Analysis of T0 transgenic plants

PCR analysis: PCR analysis of primary transformants (T0) was conducted using CMV-CP, CMV-MP and CMV-OVG-specific primers. The PCR analysis conducted on the CMV-CP primary transformants revealed the presence of the *CP* gene in ~70% of plants (22/32), whereas the untransformed *N. tabacum* was scored PCR negative (acted as controls). In Fig. 4A, 11 out of the 22 positive transformants are represented. The PCR analysis produced a ~760bp band when electrophoresed and visualised via UV transillumination.

The PCR analysis was also conducted on the MP and OVG wild type and mutant lines. A combination of wild type and mutant *MP* putative transgenic lines were selected for analysis via PCR using specific primers that were designed for the *MP* gene as stated in materials and methods. Eight lines were selected for PCR analysis. The electrophoretic analysis of the PCR product showed that all lines analysed contained a ~870bp band (Fig. 4B).

Similarly, nine wild type and mutant lines of the *OVG* gene were analysed. A ~200bp band was observed in all transgenic lines selected. The ovg4 and ovg4.1 produced very faint bands (Fig. 4C).

Southern analysis: Genomic DNA (15 µg) was extracted from four transformants that were randomly selected from the wild type and mutant lines of CP, MP and OVG. The DNA was digested with *Hin*dIII, electrophoresed and transferred to nylon membranes and probed with their respective genes. Figure 5A illustrates positive results of CMV-CP gene incorporation into the genome of the lines screened (CP1, CP3, CP8 and CP11). The Southern results show that these lines have between two (CP8) and five (CP1) copies of the gene in the genome. As for the CMV-MP gene, the randomly selected lines (MP1, MP2, MP6 and MP7) showed between one (MP1) and nine (MP2)

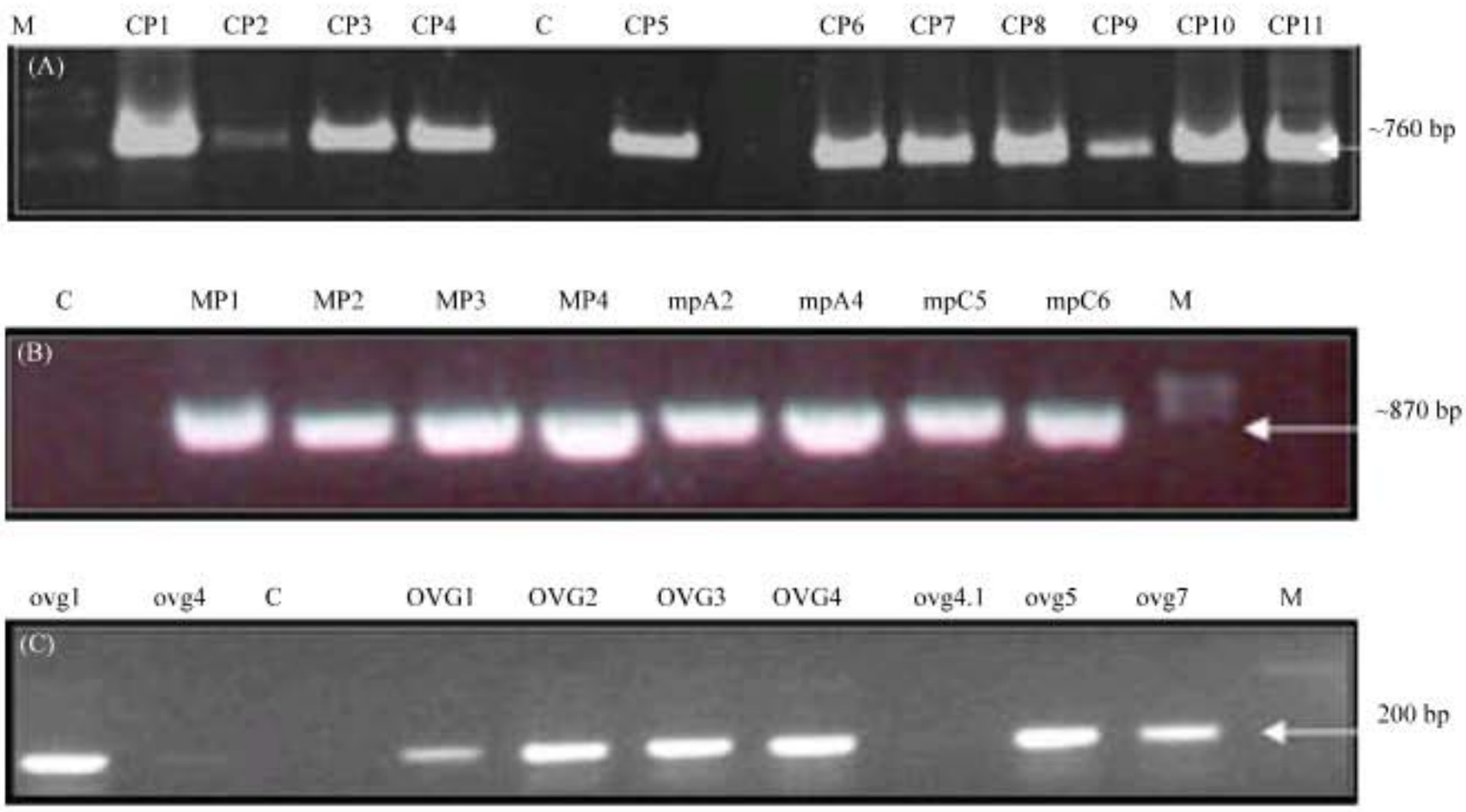

Fig. 4: PCR Analysis of Primary Transformants (T0). (A) 11 lines were randomly selected and analysed using CMV-CP Specific Primers. Lanes 1-4, 5, 6-11 are results obtained from the putative transformants. (B) Eight lines were randomly selected and analysed with CMV-MP Specific Primers. Lanes 2-9 are results obtained with the specific primers. (C) Nine lines were selected for analysis with CMV-OVG primers. Lanes 1-4, 5, 6-11 exhibit the results obtained with these transformants. C denotes controls; i.e. untransformed plants and M is the molecular marker used for determination of band size

copies (Fig. 5B) of gene in the genome. On the other hand, CMV-OVG lines (OVG1, OVG5, OVG6 and OVG8) that were selected showed two and three copies of the gene (Fig. 5C).

Mutants of the MP and OVG transformants were also randomly selected for PCR analysis. Figure 5D shows the presence of the *mp* gene in the genome. There were between 2 to 4 copies of the gene incorporated in the genome of the randomly selected lines (mpA2, mpA5, mpC3 mpC6). The *mpA* was the mutant *MP* gene with a site-directed mutation in codon 70 and *mpC* has a site-directed mutation in codon 90. Figure 5E has the results obtained from analysing four ovg mutant lines (ovg1, ovg4, ovg 5 and ovg7.8). The results show that there are between two and six copies of the gene incorporated into the genome. These lines were also screened via northern blot to determine if the transgene was being expressed. All lines showed expression (data not shown).

Evaluation of virus resistance in T1 transgenic lines: To analyse the degree of resistance against CMV infection; CP8, MP6, OVG1, mpC3 and ovg1 lines (five independent events), which showed two copies of either the *CP*, *MP*, *OVG* or mutant *MP* and *OVG* genes in their genome were scored for maximum seed setting in the T0 generation. Good seed setting lines were selected for screening. The seedling progeny from the T0 generation of all the five lines were self-fertilized and ~60 seeds from each line was germinated aseptically on MS kanamycin (100 mg L^{-1}) medium. The percentage of survival was analysed in these plants. The results indicate that the progeny of the five lines segregated with a ratio of ~9:3:3:1, thus suggesting that two copies of the gene was in each line. From the seedlings generated in the T1 generation of CP8, MP6, OVG1, mpC3 and ovg1 lines, only 40, 46, 26, 35 and 36 seedlings, respectively survived on kanamycin. These seedlings were challenged for resistance to CMV infection. Inoculation of 7-8-week-old progenies at a 1:10, 3:10 and 5:10 concentration resulted in chlorosis and mosaic symptoms in all the non-transformed control plants between 1-4 days in the inoculated leaves. In case of serious viral infections (when high inoculum was applied) these plants died in ~2 weeks.

The transgenic CP8, MP6, OVG1, mpC3 and ovg1 lines did not show any symptoms on the inoculated leaf in the first 7 days after post-inoculation. Broadly, the development of response in all five lines could be categorised into five: resistant, moderately resistant, low resistance, susceptible and highly susceptible. Plants showing moderate resistance were those that consisted of

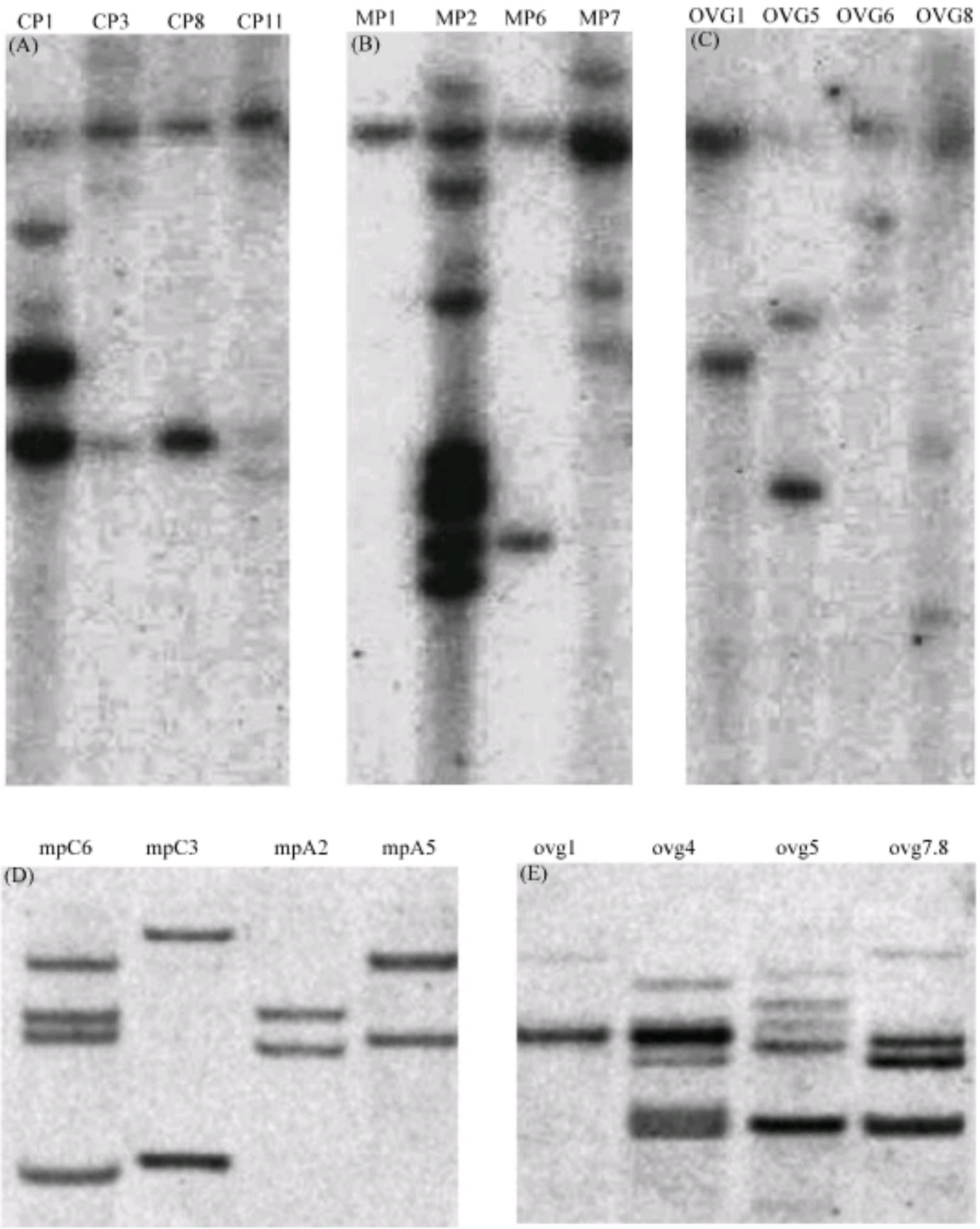

Fig. 5: Southern blot of T0 transgenic lines showing the integration of wild type and mutant genes when probed with their respective genes (*CP*, *MP* and *OVG*). DNA (15 mg) was digested with *HindIII* and electrophoresed on a 1.0% agarose gel. DNA fragments were transferred onto nylon membrane (Hybond N +) and hybridized with the probe DNA labelled with 32P*a*–dCTP using the Megaprime DNA labeling kit (Amersham Pharmacia Biotech). Four (A) CP wild type (B) MP wild type (C) OVG wild type (D) mp mutant and (E) ovg mutant lines were randomly selected from the total lines generated in this study

symptomatic and asymptomatic plants in varying proportions (Table 3). As seen in Table 3, all five lines showed 70-90% complete resistance (10 plants per test group) when inoculated with 1:10 concentration of virus. At 3:10 and 5:10 concentration, all five lines showed 40-70% and 0-30% resistance respectively. The five lines showed susceptibility when inoculated with 5:10 concentration of virus. All lines showed 10-50% susceptibility. The higher levels of susceptibility were only seen when high viral inoculum was used i.e., 5:10 concentration. The control untransformed plants had higher levels of susceptibility, ranging from 40-70%. One progeny from each line (CP8/9, MP6/2, OVG1/5, mpC3/3 and ovg1/9) that showed complete resistance to CMV infection when evaluated with the naked eye was assessed for virus accumulation through northern blot assays using the uninoculated, inoculated and upper leaf of each plant line. The northern assays were done at 8 dpi. The results revealed that although there was an increase in the levels of virus accumulation in the inoculated

Table 3: Disease severity and percentage of resistant and susceptible plants in the transgenic and control lines 20 days post-inoculation

Line	Disease severity[b]					Percentage		
(Line-treatment ratio)[a]	1	2	3	4	5	Resistant-1	Intermediate resistance (2+3)	Susceptible (4+5)
CP8-1	9	1	-	-	-	90	10	-
CP8-2	7	1	2	-	-	70	30	-
CP8-3	3	4	2	1	-	30	60	10
MP6-1	9	1	-	-	-	90	10	-
MP6-2	5	2	1	2	-	50	30	20
MP6-3	3	4	2	1	-	30	50	20
OVG1-1	9	1	-	-	-	90	10	-
OVG1-2	4	3	2	1	-	40	40	20
OVG1-3	3	1	3	2	1	30	40	30
mpC3-1	7	2	1	-	-	70	30	-
mpC3-2	5	2	2	1	-	50	40	10
mpC3-3	2	1	2	3	2	20	30	50
ovg1-1	7	2	1	-	-	70	30	-
ovg1-2	4	3	2	1	-	40	50	10
ovg1-3	2	4	3	1	-	20	70	10
Control-1	-	2	4	3	1	-	60	40
Control-2	-	1	4	3	2	-	50	50
Control-3	-	1	2	3	4	-	30	70

[a]Treatment ratio: 1 = 1:10; 2 = 3:10 and 3 = 5:10, [b]1: Resistant, 2: Moderate resistance, 3: Low resistance, 4: Susceptible, 5: Highly susceptible

leaves (I) of plants (Fig. 6 A-F), the systemic leaves (upper leaves) showed lower levels (significantly at times) of virus accumulation (Fig. 6A-F). The level of virus accumulation in the upper leaves was found to be at least ~2-3 times lower compared to the inoculated leaves (Fig. 6A-F). The lowest level of virus accumulation was observed in the CP8/9 followed by MP6/2 and OVG1/5.

The lines that showed complete resistance were re-inoculated with the viral inoculums (1:10). For this purpose, only the CP8 and MP6 lines were used as they exhibited the highest level of resistance and showed no visible symptoms. The inoculated and systemic leaves were used to analyse the viral RNA levels in the plants at 8 dpi. The same probes used to test the T2 lines were used in assaying the T3 generation of these two lines. Despite the differences in the virus accumulation levels, no phenotypic differences could be identified between the two lines. The major difference found in the T3 analysis is that the RNA 3 and 4 levels where higher then RNA1 and 2. These lines were analysed again at 25 dpi (Fig. 7). The non transformed control showed accumulation of all four CMV RNA species.

In this study three different genes, *MP*, *CP* and *OVG*, were isolated from *Cucumber mosaic virus* infected plants via RT-PCR. The genes were then used to produce wild type and mutant constructs of the above genes. The T0-transformed *N. tabacum* plants were analysed by PCR, Southern and, northern analyses. These assays confirmed the presence of the CMV-CP, CMV-MP, CMV-OVG and mutants *MP* and *OVG* genes in the plant genome. The Southern blot analyses showed that some of these lines had several copies of gene incorporated into the genome. However, through this study we noticed that there was no correlation between the copy numbers of the plant and the RNA accumulation levels. Higher copy numbers did not result in lower levels of RNA accumulation within plants. This would therefore allude that the location of transgene incorporated within the genome will determine the effectiveness of RNA level inhibition (positional effect) (Geyer *et al*., 2007; Peretz *et al*., 2007).

The T1 lines carrying the wild type and the mutant genes of *MP* and *OVG*, were used in the analysis of virus accumulation at different times (dpi) and from different positions of the leaf related to inoculation site (inoculated leaf and/or upper leaf). The results indicated that these lines produced near-complete resistance to complete susceptibility. Primarily, all resistant transgenic plants (>50%) exhibited delayed symptom development as reported in a number of studies (Nakajima *et al*., 1993; Gielen *et al*., 1996). A few strongly resistant plants were also obtained where no symptoms developed even as late as 30 dpi. The virus accumulation in transgenic and untransformed plants were analysed in the T2 generation of five lines (CP8, MP6, OVG1, mpC3 and ovg1) that showed two copies of either the *CP*, *MP*, *OVG* or mutant *MP* and *OVG* in their genome and scored maximum seed setting in the T0 generation. In the northern assays using the 3' conserved region of all four RNA of CMV, it was observed that the level of virus accumulation was highest in the control plants and lowest in the upper leaf (systemic studies). The infected leaves of the transgenic lines showed lower levels of viral RNA as compared to the untransformed control. Similar observations were made by Okuno *et al*. (1993) and Reimann-Phillipp (1998).

According to Reimann-Phillipp (1998), a reduced rate of virus accumulation in inoculated leaves and slower systemic spread are frequently observed in transgenic CP-accumulating plants owing to slower replication rates

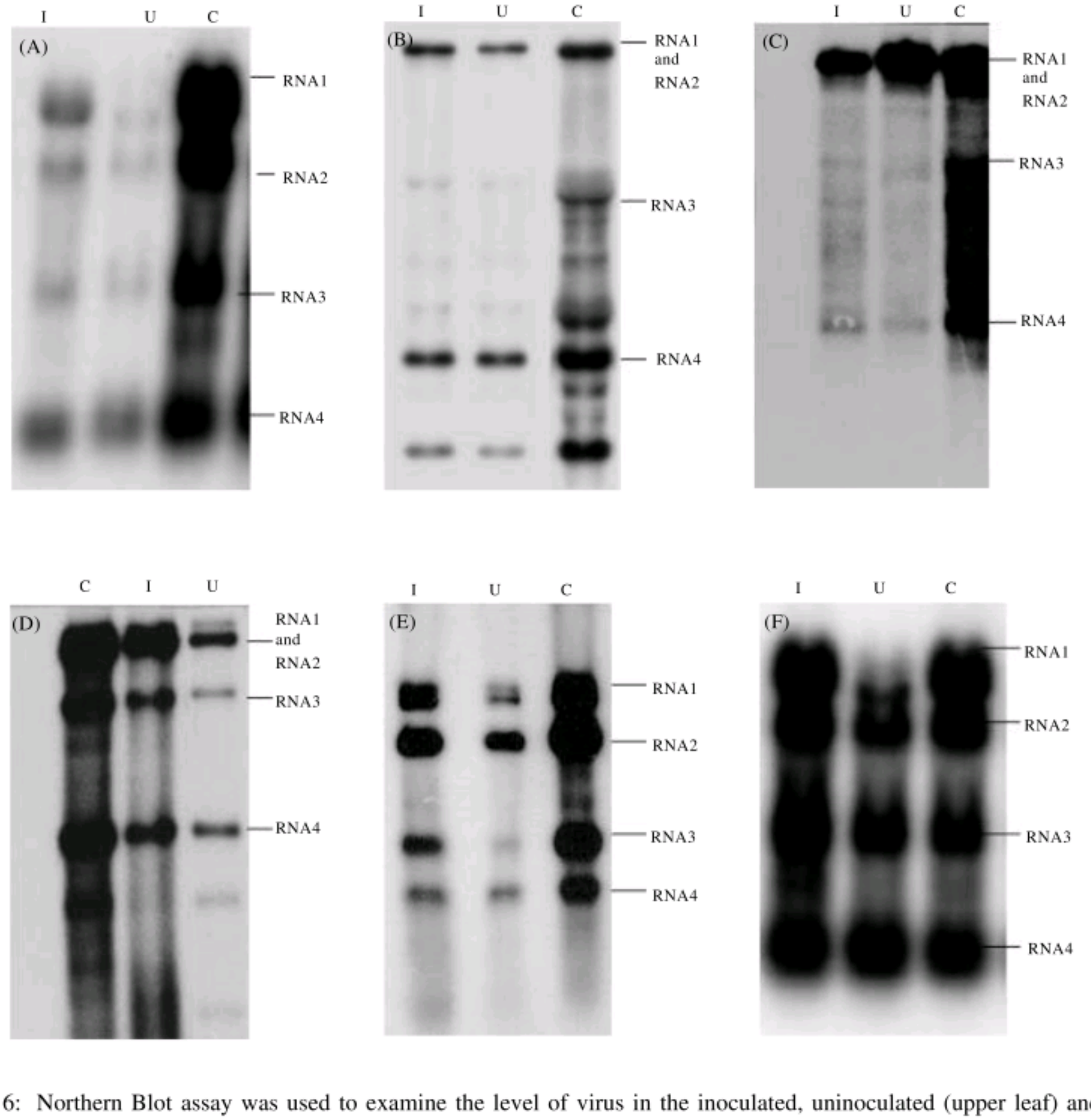

Fig. 6: Northern Blot assay was used to examine the level of virus in the inoculated, uninoculated (upper leaf) and untransformed plants of the T2 generation of 5 transgenic lines (CP8/9, MP6/2, OVG1/5, mpC3/3, ovg1/9) in *Nicotiana tabacum*. Total RNA was electrophoresed on a 1.0% agarose gel. The RNA fragments were transferred onto nylon membrane (Hybond N +) and hybridized with a probe that contained the conserved sequence in the 3'end of RNA1-4. The probe was labeled using the 32P*a*–dCTP Megaprime DNA labeling kit (Amersham Pharmacia Biotech). Here we assayed the viral levels in 5 progenies: (A) CP8/9, (B) MP6/2, (C) OVG1/5, (D) mpC3/3, (E) ovg1/9 and (F) untransformed plants. I denotes the RNA obtained from virus inoculated leaf, U is the RNA from the upper leaf and C is for the RNA from untransformed plantlets

or interference with local or systemic virus transport. Higher accumulation of CMV in inoculated leaves but no systemic spread may be due to interference with either entry into the phloem or vascular long-distance transport as suggested earlier by Taliansky and Garcia-Arenal (1995). Kim and Palukaitis (1997) also reported similar occurrence with other CMV related genes such as the movement protein. In lines carrying the movement protein gene, the accumulation of movement protein from the virus bound to the antisense movement protein within the

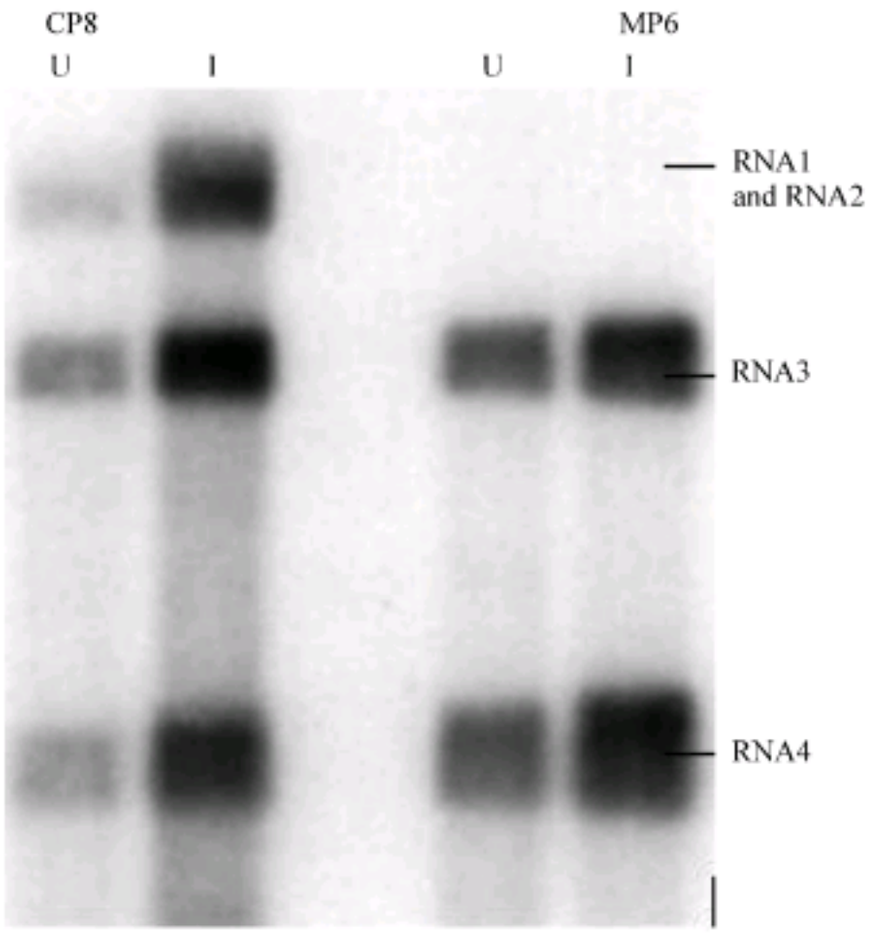

Fig. 7: Northern Blot assay was used to examine the level of virus in the inoculated, uninoculated (upper leaf) and untransformed plants of the T3 generation of 2 transgenic lines CP8 and MP6 in *N. tabacum*. Total RNA was electrophoresed on a 1.0% agarose gel. The RNA fragments were transferred onto nylon membrane (Hybond N +) and hybridized with the probe that contained the conserved sequence in the 3'end of RNA1-4. The probe was labeled using the 32P*a*–dCTP Megaprime DNA labeling kit (Amersham Pharmacia Biotech). I denotes the RNA obtained from virus inoculated leaf, U is the RNA from the upper leaf

transgenic host and therefore became ineffective (Cuozzo *et al*., 1988). This resulted in the interference of systemic transport of the viral genome across the cell within the plant. The overlapping gene however is a new gene which functions in a similar manner to the triple block proteins in certain viruses (Ding *et al*., 1994; Hellwald *et al*., 2000; Dohi *et al*., 2002; Sahidatul *et al*., 2008). Earlier study has shown that this gene is involved in the disease process of CMV. However the mechanism by which this gene is able to arrest the disease remains to be elucidated. Lines that did not show symptoms after initial inoculation, were back inoculated and observed further at 8 and 25 dpi. In these lines it was observed that the level of RNA3 and 4 accumulated at higher levels then RNA1 and 2. We are unable to explain this difference at this point.

The main objective of this study was to determine whether the pathogen-mediated resistance strategy could be applied in producing resistant transgenic plants that were resistant towards CMV infections. Three viral genes were selected and produced via RT-PCR from a Malaysian CMV isolate. This is the first study where all three gene constructs and *MP* and *OVG* mutant constructs were used to compare and contrast the ability of these genes to afford pathogen-mediated disease resistance towards CMV. In addition, we have also generated *OVG* and *MP* mutant genes. Previous studies have mostly been directed towards individual wild type gene studies (Fitchen and Beachy, 1993). Through this study we found that the *CP* and the *MP* genes were better candidates for disease resistance compared to the *OVG* gene. This is illustrated clearly from the lower level of RNA accumulation seen in both these constructs as compared to the *OVG* gene. In addition, the deformity levels observed in the *OVG* transgenic lines and the higher mortality rate of these plants when moved into the glasshouse for acclimatisation, did not favour the use of this transgene in future research.

The transgenic lines were also tested using 3 different concentrations of inoculums. In previous studies, 1:10 concentration of inoculum was used. The ability of some of these lines to withstand high levels of inoculum (5:10) was indicative that some of these lines exhibited good levels of disease resistance. Given this observation, we believe that the constructs that were made in this study may have the potential to be applied in commercially important crops in which CMV causes drastic reduction of yield and quality (Tomlinson, 1987). In our future research, we propose to study the effectiveness of this construct in other CMV susceptible products in Malaysia, such as chilli, tomato and brinjal.

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