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Transformation of Overlapping, Movement Protein and Coat Protein Gene into *Nicotiana tabacum*

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A study was carried out to transform overlapping (*OVG*) gene, movement protein (*MP*) gene and the coat protein (*CP*) gene into *Nicotiana tabacum*. The cDNAs of all the three genes mentioned were successfully obtained. The 200 bp cDNA of the *OVG* gene was amplified via RT-PCR and subsequently used in the random mutagenesis process, to generate three mutants, *ovg*1, *ovg*4 and *ovg*5. cDNA of *MP* with an estimated size of 870 bp and its site-directed mutants, *mp*A and *mp*D were also generated in addition to the 760 bp wildtype *CP* gene obtained from a previous research. All the cDNAs were individually cloned into pPCR-Script, and subsequently sub-cloned into the plant transformation vector, pCAMBIA3301. Each of the recombinant pCAMBIA3301 plasmids, harbouring one of the above mentioned genes and mutant alleles of interest, was then transformed into *Agrobacterium tumefaciens* LBA4404. Transformation experiments into tobacco plants (*Nicotiana tabacum* cv. White Burley) were carried out by the leaf-disc co-cultivation method. Primary screening of transformants revealed an average of 39.33% putative transformants (positives) for all constructs used (59% pCAMBIA-CP and 56% pCAMBIA-OVGori and mutant *ovg*1, *ovg*4, *ovg*5 and *ovg*7 and 3% pCAMBIA-MP, mutants *mpA* and *mpC*). Further screening of the surviving transformants showed that 72% (70% pCAMBIA-CP, 90% pCAMBIA-OVGori and mutants, 56% pCAMBIA-MP and mutants) showed GUS (β-glucuronidase) activity when assayed. Integration of the transgene into the genome was confirmed via PCR and Southern-PCR methods. First generation screening of randomly selected putatively transformed plants carrying constructs of pCAMBIA3301-CP, pCAMBIA3301-OVGori and pCAMBIA3301-ovg4 were 100% positive in the PCR and Southern PCR analysis producing ~760 bp, ~200 bp and ~800 bp bands for *CP*, *OVG* and *gus* genes respectively. Further testing was carried out via Southern-DNA method using the same transformants. The results showed positive integration of transgene of pCAMBIA3301-CP and pCAMBIA3301-OVGori into tobacco plantlets.

Keywords*:* Overlapping gene, movement protein, coat protein, transformation, genome integration.

Cucumber mosaic virus (CMV) is a member of the family *Bromoviridae*, genus *Cucumovirus*. CMV infects more than 800 plant species worldwide, a majority of which is economically important (Smith *et al.,* 2000). As CMV spreads easily, it may therefore pose a threat to agricultural produce and could significantly affect the economy of a country that is dependent on agriculture to sustain its economy. This viral infection causes a range of effects from subtle colour changes and leaf distortion to severe plant stunting and deformity (Agrios 1997).

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The movement and replication within plants of CMV is affected by each of the five known gene products translated from the three genomic and two subgenomic RNAs. The 1a protein, a component of the viral replicase containing putative helicase and methyl transferase domains, influences the rate of systemic infection (Gal-On *et al.,* 1994; Ye *et al.,* 2009). The 2a protein, which contains the conserved RNA polymerase motifs, is a component of the CMV replicase which influences the movement of viruses in plants (Hellwald and Palukaitis, 1995; Seo *et al.,* 2009). Similarly, the 2b protein also influences movement, but in a host specific manner (Ding *et al.,* 1995). The 3a protein is required for the cell-to-cell movement of the virus and hence is also known as the movement protein (Kaplan *et al.,* 1995; Kim *et al.,* 2004) and traffics itself and the viral RNA from cell-to-cell. RNA 3 encodes two proteins, namely, the coat protein (CP), of which 3a is the translation product of RNA 3, whereas CP is translated from subgenomic RNA (RNA 4) (Ding *et al.,* 1994; Srivastava & Raj 2008). Some isolates also carry a satellite RNA of 330-405 nucleotides, affecting symptom expression and virus replication mainly in solanaceous hosts. RNA 4a is a novel subgenomic RNA derived from RNA 2 and encodes the overlapping gene of RNA 2 (called 2b). CMV 2b protein acts as a suppressor of host antiviral defense, based on its requirement for hypervirulence synergistic infections and spread of CMV in host plant infections (Ding *et al.,* 1994; 1995; 1996; Shi *et al.,* 2002; Zhang *et al.,* 2006).

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. 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 gene and another gene that is only present in CMV, which is the overlapping gene to generate transformation constructs. Since the overlapping gene has only been reported in CMV, we believe it has an important role in the development of the disease symptom in plants and therefore may contribute towards the establishment of disease resistance.

The objective of this study is to produce wildtype and mutant cDNA clones of the above mentioned CMV genes through the process of reverse transcriptase PCR and then to subclone the cDNAs into pCAMBIA3301. These constructs are 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 will then be tested for its efficacy in producing resistance and the level of resistance afforded by each line will be recorded. Here we report the constructs that were generated for the above purpose. These constructs may be used to transform any plant that is susceptible to CMV, provided a transformation system has been established for this species. We believe that some of these constructs will be of interest to farmers as it will provide a means of generating higher yield without the use of expensive, toxic and ecologically damaging chemicals (fertilizers, herbicides, or pesticides) and to reduce loss via disease

MATERIAL AND METHODS

Plant Material and Virus

Tomato, chilli and tobacco plants were planted to provide a host for virus propagation. The original virus stock was taken from Plant Biotechnology Laboratory, Universiti Kebangsaan Malaysia. Virus inoculation was conducted on 6 week old plants as described by Noordam (1973) where the virus was inoculated onto leaves dusted with Carborundum.

RNA extraction

RNA of CMV was extracted from CMV infected plants using the Guanidine Hydrochloride (GHCl) method with modification (Logermann *et al.,* 1987) (Guanidine Thiocyanide (GTC) was used to replace GHCl).

Reverse transcriptase PCR

cDNA clones were obtained from the extracted RNA using Access RT-PCR Introductory System (Promega, USA) kit. This is a two-step RT-PCR method-using specifically designed forward and reverse primers (Table 1). The design of the primers was based on the sequence (code CAA25494 GI59044) in NCBI database (http://www.ncbi.nlm.gov/). The RT-PCR gene products were ligated into the pPCR-Script using Quick

Ligation kit (QIAGEN, USA) and sequenced.

Mutagenesis of genes

Mutagenesis on the *OVG* gene was carried out using GeneMorph® Random Mutagenesis Kit (Stratagene, USA) to obtain mutants of the wildtype genes. Primers used in the process were designed using Primer Premier 5 (Premier Biosoft International, C.A.). The primer sequences (Table 2) recommended by the software were later adjusted manually to contain suitable restriction sites for the subsequent cloning activities in pCAMBIA3301. Mutants for *MP* gene are taken from previous work using Site-Directed Mutagenesis Kit (Promega) (Nadarajah *et al.,* 2003). The primers used were to produce mutation on codon-70, mpA and codon-90, mpC (Table 3).

Construct preparation and transformation into plant

Wildtype and mutant cDNA clones of *CP* gene (Shahidatul *et al.,* 2008), *MP* gene (Nadarajah *et al.,* 2003) and *OVG* genes obtained were ligated into plant transformation vector pCAMBIA3301. pCAMBIA 3301 carries CaMV35S promoter and two reporter gene *uid*A and *Bar* that facilitates the selection of positively transformed products. Constructs were transformed into DH5α competent cells using the heat shock method (Ausubel, 1998). Screening of transformants was done by antibiotics 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 0.5mg/l 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 *Agrobacterium tumefaciens.*

PCR and Southern blot analysis for transgene

Primary screening involves growing the transformed plant callus in MS medium with antibiotics selection (250 µg/ml kanamycin and 350 µg/ml carbenicillin and 40 mg/L PPT). All plant calluses were subjected to primary screening. Untransformed plant callus was used as control. 1180 calluses were tested at this stage for pCAMBIA-OVGori and its' mutants, 300 for pCAMBIA-MP and mutants and another 30 for pCAMBIA-CP wildtype gene construct.

This was followed by a secondary screening using GUS assay (Jefferson *et al.,* 1987) to confirm positive transgenic plants. Two fresh leaf discs were taken from the putative transformed plantlets and incubated for one hour to overnight at 37°C in 5-bromo-4-chloro-3-indolyl glucuronide (X-gluc) which gave a blue precipitate at the location of enzyme activity. Plantlets giving positive results are transplanted and transferred to the green house and acclimatized to the natural environment. 32 randomly chosen plantlets carrying pCAMBIA-CP, 10 carrying pCAMBIA-OVG and mutants and 9 carrying pCAMBIA-MP and mutants were tested using GUS assay. Subsequently, the positive transgenic plants were further confirmed via Southern-PCR and Southern Blotting using *DIG High Prime DNA Labeling and Detection Starter Kit II* (Stratagene, USA).

RESULTS AND DISCUSSIONS

Virus Propagation

Leaves of young host tobacco plants (*Nicotiana tabacum* var. White Burley), tomato (*Lycopersicon esculentum*) and cucumber (*Cucumis sativum*) were inoculated with dry stock of CMV. Symptoms were observed two weeks post inoculation. Mosaic patterns on the leaves can be observed on tobacco plant. Cucumber leaves showed bleaching symptoms and mosaic patterns whereas tomato plants developed "shoe-strings" symptoms. All the plants became stunted as compared to non-infected plants.

Isolation of Viral RNA from Infected Host

Leaves from the infected plants were cut and used for RNA extraction. The viral RNA was obtained and used in obtaining the cDNA clones for each gene construct (movement protein, coat protein and overlapping gene) using the RT-PCR technique. An approximate ~ 0.20 kb product was amplified 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/). Result 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 with the sequence reported by Ding *et al.,* 1994 for the CMV overlapping gene (CAA25494 GI59044).

cDNA of *OVG* 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 using GeneMorph® Random Mutagenesis (Stratagene, USA). Random mutagenesis was chosen due to the lack of information on the important locations of the sequence as compared to the other genes such as movement protein (MP) and coat protein (CP).

Mutants of the *MP* gene was produced using the site directed mutagenesis method as described in earlier publications (Tan *et al.,* 2002; Nadarajah *et al.,* 2003). The two sites chosen for mutagenesis are codon-70 and codon-90.

Table 1. Forward and Reverse Primers Designed for RT-PCR of *OVG* gene

Forward Primers Identity	Sequence	Size
SD6F	5'-ACG GAT CCT GGT CTC CTT ATG GAG AAC CTG TGG-3' BamHI	33-mer
SD9F	5'-GGG ATC CTT TTG TAT ATC TGA GTT CCG GG-3' BamHI	29-mer
SD10F	5'-GTC CAA GAG GTA TTC CTC TGG ATC C-3' BamHI	25-mer
Reverse Primers Identity	**Sequence**	**Size**
SD7R	5'-AGG ATC CAT GGA TGT GTT GAC AGT AGT GG-3' BamHI	29-mer
SD8R	5'-CAC GCG TAA AAC ATA TAG ACT CA-3' MluI	23-mer
SD11R	5'-CAA AAG CTT TAG ACT CA-3' HindIII	17-mer
SD12R	5'-CAA AAC ATA TAG AAT TCA AGG-3' EcoRI	21-mer

Table 2. Forward and reverse primers designed for random mutagenesis of *OVG* gene

Identity	Sequence	Size
Fovg 1	5'– CCGGGTCGACCGTCGAACTAGAGTTAGGC-3' SalI	29-mer
Rovg 1	5' –CCCTGCCTCCTCTGTGAATCTAGACTTG –3' XbaI	28 - mer

Table 3. Primers used in Site-Directed Mutagenesis for *MP* gene

Identity	Sequence	Size
mpA	5'GCC CTG AAG TCA TTA A***AT GCA T***GG C-3' *Nsi*I	25
mpC	5'GGA T***GC GGG C***TG ATA AAG CTA TT-3' *Bss*HII	25

These two sites are located in a highly conserved region and codon-90 has been shown to play an important part in the assimilation and movement of protein MP from cell to cell (Boccard & Baulcombe 1993). The product size was approximately 870 bp and Figure 1 has the full sequence of the *MP* gene and the mutated codons are highlighted in bold.

cDNA for coat protein (product size ~760 bp) of CMV was obtained from Hanina (1999), Universiti Kebangsaan Malaysia. All the cDNAs for *OVG* wildtype, *ovg1, ovg4, ovg5, ovg7*, *MP* wildtype, *mpA, mpC* and *CP* wildtype were ligated into plant transformation vector pCAMBIA3301. Figure 1 shows the sequence of the original cDNA of *OVG* gene obtained via RT-PCR. The mutants obtained from random mutagenesis for *OVG* gene are as listed in Figure 2. *ovg*1, *ovg*4, *ovg*5 each has two nucleotides changed while *ovg*7 has a single nucleotide change. There were also two constructs that were built with a combination of more than one cDNA gene. These are pCAMBIA3301-CP-OVG and pCAMBIA3301-MP-OVG.

pCAMBIA3301 was chosen as a plant transformation vector as it has the marker gene *bar*, *NPTII* and a reporter gene *gus*A which enables easy screening of transformant. The constructs obtained were transformed into *Agrobacterium tumefaciens* LBA4404 through heat-shock method (Ausubel 1998). Sterile cut tobacco leaves were co-cultivated with *A. tumefaciens* carrying the recombinant plasmid and acetosyringone produced naturally by the cut tobacco leaves stimulate the expression of *vir* genes.

Morphology of the regenerants

The growths of transformed plantlets were much slower as compared to untransformed plantlets. The slower growth and differences in the physiological development was suspected to be caused by the long term exposure to the herbicide selection media (PPT - phosphinothricyn), carbenicilin and kanamycin. Transformed plantlets also showed a less developed root system as compared to untransformed plantlets (Figure 3A). Despite the morphological differences, the plantlets are still able to grow healthily albeit slower than non-transformed plantlets. It has been shown by Yepes & Aldwinckle (1994) that the antibiotic carbenicillin induced callus formation and but it resulted in retarded root formation.

All the putative transformants carrying the constructs pCAMBIA-MP, pCAMBIA-CP and pCAMBIA-OVGori are morphologically normal. The putative transformants carrying the mutant constructs pCAMBIA-ovg1, pCAMBIA-ovg4,

Table 4. Primers that were designed for use in the PCR analysis of transgenes

Forward primers Identity	Sequence	Size
Fmp1	5'-GAT T**AA GCT T**GC ATG GCT TTC CAA GGT ACC AG-3'	32-mer
Sze 1	*Hind*III5'-GAT **TTC TAG A**GC ATG GCT TTC CAA GGT ACC AG-3'	32-mer *Xba*I
coat1	5'-CTC GAA TTC **GGA TCC** GCT TCT CCG CGA G-3' *BamH*I	28-mer
Gus F	5'-CGC CGA TGC AGA TAT TCG TA-3'	20-mer
Reverse primers Identity	**Sequence**	**Size**
Sze 1R	5'-C**GG ATC C**GC TCA AGA CCG TTA ACC ACC TGC-3'	30-mer
Rmp1	*BamH*I5'-C**GT CGA C**GC TAA AGA CCG TTA ACC ACA TGC-3' *Sal*I	30-mer
Rmp2	5'-C**GT CGA C**GC TCA AGA CCG TTA ACC ACC TGC-3' *Sal*I	30-mer
coat2	5'-GGC GAA **TTC GAG C**TC GCC GTA AGC TGG ATG GAC-3' *Xba*I	33-mer
Gus R	5'-ATT AAT GCG TGG TCG TGC AC -3'	20-mer

(A)

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GTAATCTTAC CACTTTCTTT CACGTCGTGT CGCGTCAGTC CACGCTGTGT GTGTGTGTGT  60
GTTAGTTAGT GTGTCGTGTT TAGATTACGA AGGTTATGGC TTTCCAAGGT ACCAGTAGGA 120
CGTTAACTCA ACAGTCCTCG GCGGCGTCGT CTGGCGACTT ACAGAAGATA TTATTCAGCC 180
CCGATGCCAT CAAGAAGATG GCTACTGAGT GTGACCTAGG TCGACATCAT TGGATGCGCG 240
CGGATAACGC CATCTCTGTC AGACCTCTCG TTCCCCAAGT AACCAGTAAC AATTTATTGT 300
CTTTCTTTAA ATCTGGGTAT GATGCCGGTG AATTGCGCTC TAAAGGCTAT ATGAGCGTTC 360
CTCAAGTGCT GTGTGCCGTT ACCAGGACGG TTTCTACGGA TGCTGAGGGT TCTTGGAAAA 420
TTTATTTGGC TGACCTAGGT GATAAAGAAT TATCCCCAAT TGATGGGCAG TGTGTTACTT 480
TACATAACCA TGAGCTCCCT GCTTGGAGAT CTTTCCAACC TACCTACGAT TGCCCCATGG 540
AATTAGTTGG TAATCGGCAT CGGTGTTTCG CGGTAGTCGT TGAGAGACAT GGTTATATTG 600
GTTACGGTGG TACCACTGCT AGCGTGTGTA GTAACTGGCA AGCTCAGTTT TCTTCAAAGA 660
ATAATAATTA CACACACGCC GCTGCTGGTA AGACTCTTGT GTTGCCTTAC AACAGATTAG 720
CTGAGCATTC GAAACCGTCA GCCGTCGCTC GCCTGTTGAA GTCGCAGTTA AACAACGTTA 720
GCTCATCGCG CTATCTTTTG CCGAACGTTG CTCTTAATCA AAATGCGTCT GGGCACGAGT 840
CCGAGATTTT AAACGAAAGC CCTCCCATCG
```

(B)

***mp*A (mutation on the 70th codon)**

```
181 – CCG ATG CCA TCA AGA AGA TGG TGC CCT GAA GTC ATT AAA TCG ATG GCG
       P   M   P   S   R   R   W   C   P   E   V   I   K   S   M   A
      ATC GTT ACC GCC
       I   V   T   A
```

***mp*C (mutation on the 90th codon)**

```
241 – AAC GCA TTC ACA TGG ATG CGG GCT GAT AAA GCT ATT TTC CCT AAG TCA
       N   A   F   T   W   M   R   A   D   K   A   I   F   P   K   S
      CCT GTA ACA ATC
       P   V   T   I
```

Fig. 1. *MP* gene of CMV. (A) Sequence of *MP* gene from RT-PCR; (B) Sequence of mutants obtained from site-directed mutagenesis (only location of mutation sequence provided)

pCAMBIA-ovg5 and pCAMBIA-ovg7 showed abnormal phenotype. The mosaic pattern exhibited by these constructs looked like those found on CMV infected plants. From this observation we conclude that while *OVG* has only been reported thus far in CMV, this gene may not be a candidate gene for use in generation of disease resistance. It may however play a crucial role in the generation of the symptoms related to the disease.

Primary screening results

The primary screening process involved growing the transformed explants on antibiotic selection media consisting of kanamycin, and carbenicillin followed by a more stringent selection agent; herbicide (Basta® / PPT). As an active ingredient in herbicides like Basta®, PPT causes plant cells to die due to ammonia intoxication. The *bar* gene that is present in lines that have been transformed, acquire tolerance towards PPT. The *bar* gene has been shown to be an efficient marker gene in the transformation systems of *Arabidopsis thaliana* (Akama *et al.,* 1995; Bouchez *et al.,* 1993), *Raphanus sativus* L. *longipinnatus* Bailey (Curtis & Nam 2001) and *Nicotiana tabacum* (Ye *et al.,* 2003). The callus that survived on antibiotic selection were then transferred to media with 40mg/L PPT. Lutz *et al.,* 2001 showed that putative transformants are resistant up to a 100mg/L of PPT, whereas non-transformants are sensitive even when concentration are as low as 4mg/L.

GUS Assays

GUS assays were conducted on root producing plantlets that have been transferred to a new growth media. This is to prevent any false positive results caused by the residual effect of *A. tumefaciens* on the plantlets.

Results from the assays (Figure 3B) showed 70% (22/32) of the putative transformants of pCAMBIA-CP from the primary screening were positive. The pCAMBIA-OVG and its mutants (*ovg*1, *ovg* 4, *ovg*5 and *ovg*7) produced 90% efficiency in transformation (9/10 plantlets tested were positive for each constructs) and pCAMBIA-MP and the mutants lines (*mp*A and *mp*C) showed only 56% (5/9 plantlets tested were positive for each constructs) positive transformants. Seventy two percent (72%) positive results (all constructs inclusive) were obtained from GUS assays

(A)

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GGGCGAATTG GGTACCCCGG GATGGATGTG TTGACAGTAG TGGTGTCGAC CGCCGACCTC   60
CACTTAGCCA ATTTGCAGGA GGTGAAACGT CGAAGACGAA GGTCTCACGT CAGAAACCGG 120
CGAGCGAGGG GTTACAAAAG TCCCAGCGAG AGAGCGCGAT CTATAGCGAG ACTTTTCCAG  180
ATGTTACCAT TCCACGGAGT A                                          201
```

(B)

***ovg*1**

```
  1 – GGG CCA ATT GGG TAC CCC GGG ATG GAA GTG TTG ACA GTA
       P  G(A) S   P   M   G   P   T  L(L) H   A   C   H
```

***ovg* 4**

```
 41 – TTG ACA GTA GTG GTG TCG ACC GCC GAC CTC.....118 - CGT CAG AAC CCG
       A   C   H   H   H   S   T  A(A) L   G            A   V  L(P) G
```

***ovg* 5**

```
121 - CGA GCG AGG GGT TAC AAA ACT CGC AGC GAG AGA GCG CGA
       A   A   S   P   M   P  S(S) A(G) S   L   S   A   A
```

***ovg* 7**

```
181 – ATG TTA CCA TTC CAC GTA CCA GAT CCC GTG
       T   A   G   L   V  H(H) G   L   G   H
```

Fig. 2. *OVG* gene from CMV. (A) Sequence of *OVG* gene from RT-PCR; (B) Sequence of mutants obtained from random mutagenesis (only location of mutation sequence provided)

In conclusion, the results show that the plant transformation technique used in this research was successful. The transformants obtained are now being tested for resistance against virus infections in the field. The infectivity assays will help us see the spectrum of resistance produced by the different constructs. The difference in resistance level of the mutants will also give us a better understanding on the importance of certain sites/location of the genes used in this study.

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