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Full Length Research Paper

Transgenic plants expressing the coat protein gene of cowpea aphid-borne mosaic potyvirus predominantly convey the delayed symptom development phenotype

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Cowpea aphid-borne mosaic virus (CABMV) is a potyvirus that infects cowpea causing significant yield reduction. However, there is no durable natural resistance to the virus within the crop and genetic engineering for virus resistance was not possible because of a lack of an efficient, reliable and reproducible cowpea transformation and regeneration protocol. Coat protein-mediated resistance to CABMV was evaluated in *Nicotiana benthamiana*, a model host for the virus. The CABMV coat protein gene from a Zimbabwean isolate of the virus was optimised for expression in plants under a CaMV 35S promoter and cloned into the Hind III site of the binary vector plasmid pBI121 to result in the plasmid pBI121-CP_k. The plasmid pBI121-CP_k was used in *Agrobacterium*-mediated transformation of *N. benthamiana* leaf sections following the co-cultivation method. Regenerated plants were analysed by PCR and Southern blot hybridisation. R1 seedlings were assayed for kanamycin resistance and for presence of the coat protein and challenged with CAMBV-infected sap. Lines showing delayed symptom development were identified but no line showing immunity was identified. Delayed symptom development is significant resistance since it affords protection to the plants during the crucial early stages of development and exerts little evolutionary pressure on the virus to evolve new strategies.

Key words: Cowpea aphid-borne mosaic virus, coat protein-mediated resistance, *Nicotiana benthamiana*.

INTRODUCTION

Cowpea aphid-borne mosaic virus (CABMV) is one of the major viruses that infect cowpea with devastating results, and total crop loss has been reported in some instances (Thottappilly and Rosel, 1992). Cultivars with long lasting resistance to CABMV are not available and wide crosses are difficult to make hampering efforts to breed for CABMV resistance. On the other hand, genetic engineering approaches to virus resistance such as pathogen-derived resistance (PDR) to viruses offer an alternative approach to managing the virus problem. Pathogen-derived resistance has been very successful for many virus groups but has not been widely used to control viral diseases of importance to farmers in developing countries.

CABMV is a member of the potyviridae family of viruses, whose infections are characterized by pin-wheel inclusions (Shukla et al., 1994). The genomes of potyviruses are made up of positive sense single-stranded RNA molecules of about 10 000 nucleotides with a genome-linked virus-encoded protein at the 5' end (5' VPg) and a 3' poly-A tail (Mlotshwa et al., 2000). The complete genomic sequence of a CABMV isolate from Zimbabwe was determined (Mlotshwa et al., 2002). It consists of 9 465 nucleotides excluding the poly-A tail and 9 159 of these nucleotides are coding sequences which translates into a polyprotein of 3053 amino acids. The polyprotein is auto-catalytically cleaved into 10 proteins with the coat protein (CP) being the most C-terminal

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Table 1. Sequences of primers designed to amplify the CABMV coat protein gene.

Name of primer	Primer sequence	Targeted product
IS13 (*Bam*HI)	5'-GCG GGATCC AACA ATG TCT GAT GAA AGA CAA AAG GAA C-3'	CABMV-Z3 CP expressible sequence
IS12 (*Sal*I)	5'-GCG GTCGAC TCA CTG CCC ATG CGT CAT CC-3'	

protein.

The CP encapsidates the genomic RNA and is essential for aphid-mediated transmission, cell-to-cell and long distance movement (Shukla et al., 1994). The (ile/val)-asp-ala-gly sequence, commonly known as (I/V) DAG of the coat protein has been shown to be essential for aphid transmission (Reichmann et al., 1992; Bendahmane et al., 2007).

Use of genetically modified (GM) crops is normally considered when conventional breeding cannot yield a solution, for example, when a useful trait is not available in the germplasm, when the goal is enhanced nutritional quality or for specialty products such as vaccines. The use of GM crops will also be considered when the benefits of the technology outweigh the risks and when intellectual property rights and regulatory issues can be addressed.

Pathogen-derived resistance (PDR) also called parasite-derived protection is the resistance conveyed to a host organism as a result of the presence of a transgene of pathogen origin in the target host organism (Sanford and Johnson, 1985). The concept of pathogen-derived resistance predicts that a 'normal' host-pathogen relationship can be disrupted if the host organism expresses essential pathogen-derived genes. The initial hypothesis was that host organisms expressing pathogen gene products at incorrect levels, at the wrong developmental stage or in dysfunctional forms, may disrupt the normal replication cycle of the pathogen and result in an attenuated or aborted infection.

Coat protein-mediated resistance (CP-MR) is the phenomenon by which transgenic plants expressing a plant virus coat protein (CP) gene can resist infection by the same or a homologous virus. The level of protection conferred by CP genes in transgenic plants varies from immunity to delay and attenuation of symptoms. CP-MR has been reported for more than 35 viruses representing more than 15 different taxonomic groups including the tobamo-, potex-, cucumo-, tobra-, carla-, poty-, luteo- and alfamo-virus groups. The resistance requires that the CP transgene be transcribed and translated.

Hemenway et al. (1988) demonstrated a direct correlation between the level of expression of the CP and the level of resistance. Most of the earlier and more detailed work on CP-MR was done with TMV (Bevan et al., 1985; Beachy et al., 1986; Powell-Abel et al., 1986; Register and Beachy, 1988; Powell-Abel et al., 1990).

The specific objective of this research project was to evaluate coat protein-mediated resistance (CP-MR) to CABMV. The model host of CABMV, *N. benthamiana* was used since no efficient, reliable and reproducible cowpea transformation and regeneration protocol is available. It must be noted however that more recently Higgins and co-workers have developed a transformation protocol that works although the level of efficiency still needs to be increased (T. J. Higgins, Commonwealth Scientific and Industrial Research Organization, CSIRO, Canberra, Australia; Personal communication).

MATERIALS AND METHODS

Chemicals, reagents and plant materials

Reverse transcriptase, *Taq* DNA polymerase, dNTPs and restriction endonucleases such as *Eco*RI, *Bam*HI and *Not*I were obtained from Roche Molecular Biochemicals (Mannheim, Germany). Other chemicals such as Trizma base, sodium acetate, potassium acetate, were obtained from Sigma-Aldrich (UK).

N. benthamiana was obtained from Prof. Richard Allison at Michigan State University, USA. Plasmid vector pCa2Nos was a kind donation from Prof. Thierry. Candresse, INRA-Bordeaux, Cedex, France. Procedures involving manipulation of genetically modified plants were carried out in containment facilities at the tobacco research board, Harare, Zimbabwe.

Cloning and sequencing of the 3' terminal region of CABMV

Total RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR) to amplify the 3' terminal region of CABMV was carried out as described by Sithole-Niang and coworkers (1996), using oligo dT primer (5'CGG GAT CCT TTT TTT TTT TTT TTT TTT T-3') and a primer based on a conserved potyviridae replicase sequence (5'GAC GAA TTC TG(T/C) GA(T/C) GC(T/G/C) GAT GG(T/C) TC-3') which incorporates restriction endonuclease recognition sites *Bam*HI and *Sal*I. The 1.2 kb fragment was recovered from gel using the GIBCO BRL Agarose gel extraction Kit according to the manufacturer's recommendations (GIBCO BRL, Madison, USA), cloned into the pGEM-T vector and sequenced.

Constructs for plant transformation

The primer pairs shown in Table 1 were designed to introduce the consensus sequence for optimum translation in plants and used to amplify the CABMV isolate 3 (CABMV-Z3) CP genes for use in making the expressible pGEM-CP_k construct.

The PCR was set up as described by Sithole-Niang et al., (1996) with the 1.2 kb pGEM-CPRep clone as template and appropriate primers (Table 1) and *Taq* DNA polymerase were used. The PCR cycle conditions were as follows: initial denaturation was at 94°C for 30 s followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 30 s and extension at 72°C for 30 s. A final extension at 72°C was carried out for 2 min before storage at 4°C.

The amplified genes were digested with *Bam*HI and *Sal*I, and ligated into similarly digested plasmid pCa2Nos, which contains a

Table S1. The sequences of primers that were designed to amplify the core region of the CABMV CP core region.

CP_{core}P1 (*Bam*HI)	5'-CGC **GGATCC** AACA ATG GAT CTG TAT AACA CCA GAG CA-3'	The core region of the CABMV CP gene
CP_{core}P2 (*Sal*I)	5'-CCC **GTCGAC** TCA AAG CAT ACC TTG CC-3'	

CaMV 35S (35S) promoter with a double enhancer and nopaline synthase (Nos) terminator. The ligated DNA was used to transform competent *E. coli* DH5α cells. Plasmid DNA was isolated from the transformants using the alkaline lysis method (Sambrook et al., 1989) and screened by restriction endonuclease digestion to identify recombinant pCa2Nos plasmids. The 35S-CP_k-Nos fragments were excised from pCa2Nos-CP_k plasmids by *Hin*dIII digestion and ligated into the unique *Hin*dIII site of the binary plasmid pBI121. The ligated DNA was used to transform competent *E. coli* DH5α cells and the plasmid DNA isolated from the transformants was screened by restriction endonuclease digestion to identify recombinant pBI121 plasmids.

Distinct colonies were picked from a plate and used to inoculate 10 ml of 2YT or LB medium supplemented with 100 mg/l ampicillin and grown overnight at 37°C with shaking. About 1.5 ml of the overnight culture was pelleted by centrifugation at 7 000 rpm for 2 min. Depending on the intended use of the plasmid DNA and availability of reagents, plasmid DNA was isolated following the alkaline lysis method (Sambrooks et al., 1989) or using Wizzard TM Plus DNA purification System (Promega Life Science, Madison, USA), according to the manufacturer's instructions.

Transformation of *Agrobacterium tumefaciens*

A. tumefaciens strain LBA 4404 was grown in 50 ml YEP medium (10 g yeast extract, 10 g bacto peptone, 5 g sodium chloride, per litre) at 28°C shaking at 220 rpm until the OD_{600} had reached about 0.5.

The cells were pelleted by centrifugation at 5000 rpm for 5 min, and re-suspended in 10 ml of 0.15 M sodium chloride. The cells were pelleted again by centrifugation at 5 000 rpm for 5 min and re-suspended in 1 ml of ice-cold 20 mM $CaCl_2$ and stored at -80°C in 200 µl aliquots. One microgram of pBI121 vector control or pBI121-CPk DNA was added to 200 µl of bacterial cells in an Eppendorf tube and incubated on ice for 30 min, transferred to liquid nitrogen for 1 min (or -80°C for 5 min), and then thawed in a 37°C water bath for about 5 min. About 1 ml of YEP medium was added and incubated at 28°C for 2 - 4 h with gentle shaking. The cells were pelleted by centrifugation for 1 min, re-suspended in 100 µl of YEP medium and plated on YEP agar supplemented with 50 mg/l kanamycin and 50 mg/l rifampicin.

Plasmid isolations were carried out following the alkaline lysis method to verify transformants before proceeding to use transformed cells in plant transformation experiments.

Sequencing of pBI121-CP_k

The pBI121-CP_k binary plasmid DNA was isolated from *E. coli* DH5α strains using the Wizard™ Plus DNA Purification System (Promega Life Sciences, Madison, USA) according to the manufacturer's instructions and sequenced using primers IS12 and IS13 following the dideoxy dye terminator method in an ABI Prism Model 3100.

Sequencing reactions using another set of primers, CP_{core} P1 and CP_{core} P2 (Table S1) were also carried out to enable sequencing across the CP-35S promoter and CP-Nos terminator junctions and verification of part of the promoter and terminator sequences.

Transformation of *N. benthamiana* explants

Transformation of *N. benthamiana* explants was done following the co-cultivation procedure (An et al., 1987). Tobacco leaf material was sterilized by treatment with 10% bleach for 10 min and washed extensively with sterile distilled water. Leaves were cut to about 1.5 cm^2 sections and wounded several times with forceps. The sections were placed in a Petri dish to which 4 ml of callus induction medium (Murashige and Skoog (MS) medium supplemented with 2.0 mg/l naphthalene acetic acid (NAA) and 0.5 mg/l benzyl aminopurine (BAP) and about 10^8 *A. tumefaciens* cells carrying pBI121-CP_k constructs grown overnight in YEP medium were added and co-cultivated for 48 h at 28°C. The bacterial cells were washed off the explants in MS medium. The explants were dried on sterile filter paper before transfer to callus induction medium together with control uninfected leaf sections for two weeks. The calli that formed were transferred to shoot induction medium (MS medium with 0.5 mg/l BAP) containing 200 µg/ml kanamycin, 250 µg/ml carbenicillin, and if necessary, 250 µg/ml cefotaxime and then finally to root induction medium (MS medium). The seedlings were allowed to grow for another 2 - 3 weeks before being transferred to hardening trays for two weeks. The hardened tobacco plants were transferred to pots in the greenhouse and allowed to grow to maturity.

Screening of R1 generation

R1 tobacco seeds were germinated on MS plates supplemented with 50 mg/l kanamycin and incubated in a growth room for 3 weeks. The germinated seedlings were then scored for resistance to kanamycin with the resistant seedlings being green while the susceptible seedlings were white. The seedlings were then transferred to seedling trays for hardening and then finally to pots in the greenhouse. Leaf samples were collected from which DNA was isolated and used in PCRs to check for the presence of transgenes (as described below). Southern blotting experiments were also carried out using the same samples, and detection after hybridisation was by the chemiluminescent procedure (Kessler et al., 1990). After 3 weeks in the greenhouse the plants were about 15 cm tall and were challenged with sap from infected plants.

Plant genomic DNA isolation

Plant tissue weighing 100 mg or more was placed in a sterile mortar and wrapped in aluminium foil together with the pestle and incubated at -80°C for at least 30 min. Upon removal from the freezer, the tissue was quickly ground with the chilled pestle and mortar, and transferred to a chilled Eppendorf tube. For smaller quantities of soft tissue culture samples, 4 - 12 mg or 20 - 200 mg of tissue were placed in an Eppendorf tube to which 150 or 450 µl of Buffer 1 (Roche Molecular Biochemicals, Mannheim, Germany) were added respectively and ground using a hand-held micro-pestle until the solution turned green and the tissue was completely homogenized.

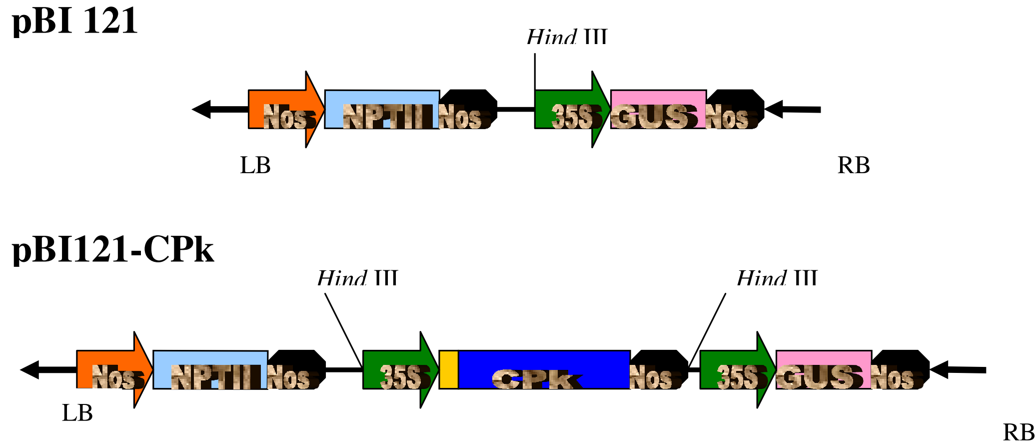

Figure 1. Plasmid maps of binary constructs used for plant transformation.

Genomic DNA was isolated from *N. benthamiana* plants either following the SDS method (Dellarporta et al., 1983) or using the plant DNA Isolation Kit (Roche Molecular Biochemicals, Mannheim, Germany).

PCR screening of transgenic plants

The PCR to screen for the presence of transgenes was set up by adding the following to a sterile 0.2 ml PCR tube, 5.0 µl DNA template (50 mg/µl), 2.0 µl primer CP_{core}P1, 2.0 µl primer CP_{core}P2, 5.0 µl 10 X PCR buffer, 3.0 µl 25 mM magnesium chloride, 5.0 µl 2.5 mM dNTP mix, 30.5 µl sterile distilled water, 0.5 µl *Taq* DNA polymerase (5 U/µl) (Table S1 for primer sequences).

The cycle was as follows; Initial melting was done at 94°C for 2 min; 35 cycles of denaturation at 94°C for 1 min, annealing at 54°C for 30 sec, extension at 72°C for 1 min; final extension at 72°C for 5 min. The amplification products were run on gel, photographed and transferred to nitrocelullose membrane.

Southern blotting

Genomic DNA of putative transgenic lines was digested with EcoRI, *Hin*dIII and XbaI in 30 µl reaction volumes consisting of 10 µg of genomic DNA, 30 U of the appropriate restriction endonuclease and 3 µl of the corresponding 10X RE buffer.

The reaction was carried out at 37°C overnight. The digested DNA was run on an agarose gel and transferred to a nylon membrane as outlined below (Sambrook et al., 1989).

After transfer, the membrane was rinsed briefly in 2X SSC and dried between Whatman 3MM paper. The DNA was fixed to the membrane by exposure to UV light for 2 min or baking at 80°C under vacuum for 2 h.

Hybridisation and detection of transgenes by the DIG chemiluminescent procedure

Clone pCa2Nos-CP or pCa2Nos-CP_{core} was digested with *Hin*dIII or BamHI/SalI. The digestion products were run on gel and the product corresponding to the CP gene was recovered using the gel recovery procedure described earlier.

One microgram of DNA to be labelled was diluted to a total volume of 16 µl with sterile distilled water and the probe labelling reaction and hybridization reactions were performed according to DIG high prime labelling and detection recommendations (Roche Molecular Biochemicals, Mannheim, Germany). The membrane was then developed with CDP-Star™ (Roche Molecular Biochemicals, Mannheim, Germany).

Excess liquid was drained and the membrane was sealed in a thin plastic bag, placed in an X-ray cassette. An X-ray film was placed over the membrane in dark and exposed for 30 s, 2 min, 5 min, 30 min and overnight periods. The X-ray films were developed in developer (Sigma Aldrich, UK) for 3 - 5 min and fixed in fixer (Sigma Aldrich, UK) for another 3 - 5 min.

Virus challenge experiments

After 3 weeks in the greenhouse the plants were about 15 cm tall and were challenged with purified virions and sap from infected plants. The challenged plants together with uninoculated control plants were then monitored for symptom development for at least 28 days, and if tolerant or resistant, allowed to grow to maturity so that seeds could be collected.

RESULTS

PCR cloning of the CABMV coat protein gene

The success of PCRs and cloning experiments were confirmed by restriction endonuclease and agarose gel electrophoresis analyses. The plasmid map of the resultant binary construct, pBI121-CP_k is shown in Figure 1.

Sequencing of CABMV pBI121-CP_k constructs

The CABMV CP-recombinant binary plasmid DNA was isolated from *E. coli* DH5α strains using the Wizard™ plus DNA Purification System (Promega, Wisconsin, USA) and sequenced using the dideoxy dye terminator method. Sequencing was carried out using primers that bind to the core region of the CP gene. The complete CP sequence shown in Figure 2 was obtained for pBI121-CP_k and verified to be correct after comparison to the

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  1 ATG TCT GAT GAA AGA CAA AAG GAA CTG GAT GCA GGT AAG GAC AAA GAC AAG GCT AAG GAA    60
  1      S   D   E   R   Q   K   E   L   D   A   G   K   D   K   D   K   A   K   E    19

 20 GCT AGA GAG CAA TCA ACG CAA CAG AAG CAA GCA AAG AAT AAA GGG GCC AAG GAA ACA GAA   120
 21  A   R   E   Q   S   T   Q   Q   K   Q   A   K   N   K   G   A   K   E   T   E    39

121  AGA GAT GTA GCA GCT AGT TCT TCA GGG CAA CTA GTC CCA CGC CTG CAG AAG ATT AGC AAA  180
 40   R   D   V   A   A   S   S   S   G   Q   L   V   P   R   L   Q   K   I   S   K    59

181  AAG ATG AAT CTT CCT ATG GTC GCT GGT AGG CTT ATC CTT AAT ATT GAT CAT TTG ATA GAA  240
 60   K   M   N   L   P   M   V   A   G   R   L   I   L   N   I   D   H   L   I   E    79

241  TAT AAG CCA AAA CAG ATT GAT CTG TAT AAC ACC AGA GCA TCA AAG GCA CAG TTC AAC ACA  300
 80   Y   K   P   K   Q   I   D   L   Y   N   T   R   A   S   K   A   Q   F   N   T    99

301  TGG TTT GAG GCC GTC AAG GAG GAG TAT GAG CTG GAT GAC GAC AAG ATG AGT GTA ATT ATG  360
100   W   F   E   A   V   K   E   E   Y   E   L   D   D   D   K   M   S   V   I   M   119

361  AAT GGT TTC ATG GTA TGG TGC ATT GAA AAT GGA ACC TCA CCT GAT GTG AAT GGA GTG TGG  420
120   N   G   F   M   V   W   C   I   E   N   G   T   S   P   D   V   N   G   V   W   139

421  ACT ATG ATG GAT GGA GAT GAG CAA GTG GAA TTT CCG CTT AAA CCC ATT GTG GAA AAC GCA  480
140   T   M   M   D   G   D   E   Q   V   E   F   P   L   K   P   I   V   E   N   A   159

481  AAA CCC ACA CTC AGA CAA GTT ATG CAC CAT TTC TCA GAC GCA GCT GAA GCG TAC ATT GAG  540
160   K   P   T   L   R   Q   V   M   H   H   F   S   D   A   A   E   A   Y   I   E   179

541  ATG AGA AAT TCC GAA GGG TTC TAC ATG CCC AGG TAT GGA CCC CTA AGG AAT TTA AGG GAC  600
180   M   R   N   S   E   G   F   Y   M   P   R   Y   G   P   L   R   N   L   R   D   199

601  AAG AGC TTG GCA AGG TAT GCT TTC GAC TTC TAC GAG GTA ACA TCC AAA ACT TCT GAT AGA  660
200   K   S   L   A   R   Y   A   F   D   F   Y   E   V   T   S   K   T   S   D   R   219

661 GCA AGA GAA GCA ATA GCA CAA ATG AAG GCC GCA GCT CTC GCC AAC GTA AAC ACC AGG ATG   720
220  A   R   E   A   I   A   Q   M   K   A   A   A   L   A   N   V   N   T   R   M    239

721  TTT GGC TTG GAT GGG AAC GTG GCA ACA GTT AGT GAG AAC ACT GAG AGG CAT ACT GCT GCT  780
240   F   G   L   D   G   N   V   A   T   V   S   E   N   T   E   R   H   T   A   A   259

781  GAT GTG AAT CAG AAC ATG CAC TCC CTC TTA GGG ATG ACG CAT GGG CAG TGA              831
260   D   V   N   Q   N   M   H   S   L   L   G   M   T   H   G   Q   *              276
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Figure 2. The nucleotide sequence of the 3' terminal 1.2 kb fragment of CABMV-Z3. The CP coding sequence is shown in bold. The possible polyprotein cleavage sites are underlined.

sequence published by Sithole-Niang et al. (1996).

Using CP_{core} P1 and CP_{core} P2 primers in another sequencing reaction, the promoter and terminator sequences and the CP-35S promoter and CP-Nos terminator junctions and part of the promoter and terminator sequences were verified against published sequences (Franck et al., 1980; Depicker et al., 1982).

Transformation of *Agrobacterium tumefaciens*

Binary plasmid DNA yield and quality from *A. tumefaciens* was poor. This DNA was used to transform *E. coli* from which good quality DNA was obtained and used to identify the clones that were still intact (Figure 3). This verification was necessary since deletions can occur in binary vectors. The verified clones were then used in plant transformation experiments. The results of kanamycin resistance and CP immunoblot assays on the regenerants are shown in Table 2.

Kanamycin resistance and CP immunoblot analyses

A total of 68 *N. benthamiana* R1 lines were tested and 42 of these were shown to be putatively transgenic since they had kanamycin resistant progeny. The lines that gave a ratio of 3 resistant to 1 susceptible include 036, 040 and 042. This ratio indicates that there is only one copy of the transferred DNA per genome. This result should be confirmed by hybridization experiments. Lines 028, 034, 035 and 049 appear to be multiple-copy lines.

PCR for transgene detection

The presence of transgenes was confirmed by PCR screening for the presence of CP gene. A PCR to detect either the entire CABMV CP gene or the CP core region was optimized and used to screen all seedlings before virus challenge experiments. Figure 4 shows a representative result of the procedure.

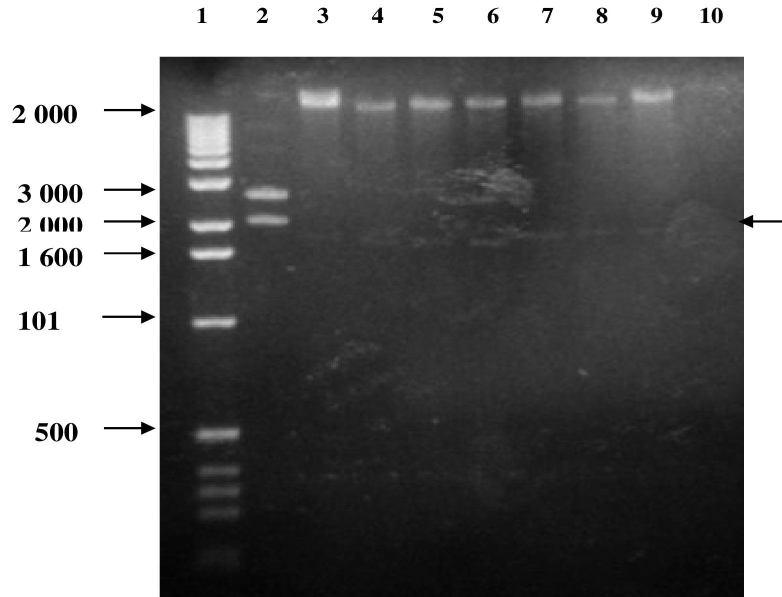

Figure 3. Photograph of a 0.8% (w/v) agarose gel of plasmid pBI121-CP_k DNA isolated from *A. tumefaciens* LBA 4404 and digested with *Hin*dIII. The lanes are: 1 – molecular weight marker, 2 – pCa2Nos-CP, 3 to 10 – DNA from different *A. tumefaciens* colonies transformed with pBI121-CP_k. The arrow shows the position of the 35S-CP_k-Nos fragment excised by *Hind*III digestion of pBI121-CP_k.

Table 2. Kanamycin resistance and CP immunodotblot analysis results.

Construct	Number of lines	Kanamycin resistant lines	CP immunodotblot +ve lines
pBI121-CP_k	(015–082) 68	016, 017, 018, 019, 020, 021, 026, 027, 028, 031, 034, 035, 036, 038, 040, 042, 045, 046, 048, 049, 051, 052, 053, 055, 057, 058, 061, 063,064, 065, 066, 067, 068, 071, 072, 073, 075, 076, 077, 078, 079, 080 42	017, 031, 035, 040, 052, 053, 055, 057, 058, 061, 064, 065, 066, 067 14
pBI121	(810-818) 9	817, 818 2	0

The PCR products were blotted onto nitrocellulose membrane and probed with the CPcore probe to confirm the identity of the amplicon (Figure 5).

Southern blotting for copy number determination

Southern hybridization using DIG chemiluminescent procedure failed to detect transgenes even though the presence of the coat protein had been demonstrated by immunoblot analysis. This is probably due to low sensitivity of the procedure. However, use of a more sensitive detection procedure such as the ^{32}P procedure might be able to detect the signal.

Virus challenge experiments

Between 7 and 42 individual plants were used per challenge experiment. Some sap-inoculated plants showed delayed symptom development, at two weeks, not within the 5 - 7 days of challenge observed for control non-transgenic plants (Table 3). One line of note is Line 035 that in addition to delayed symptom development, had symptoms that became milder with age. The plants were grown to maturity and seeds were harvested.

However, some lines were extremely susceptible to the virus and they died within 21 days of challenge, and no seed could be collected from these lines.

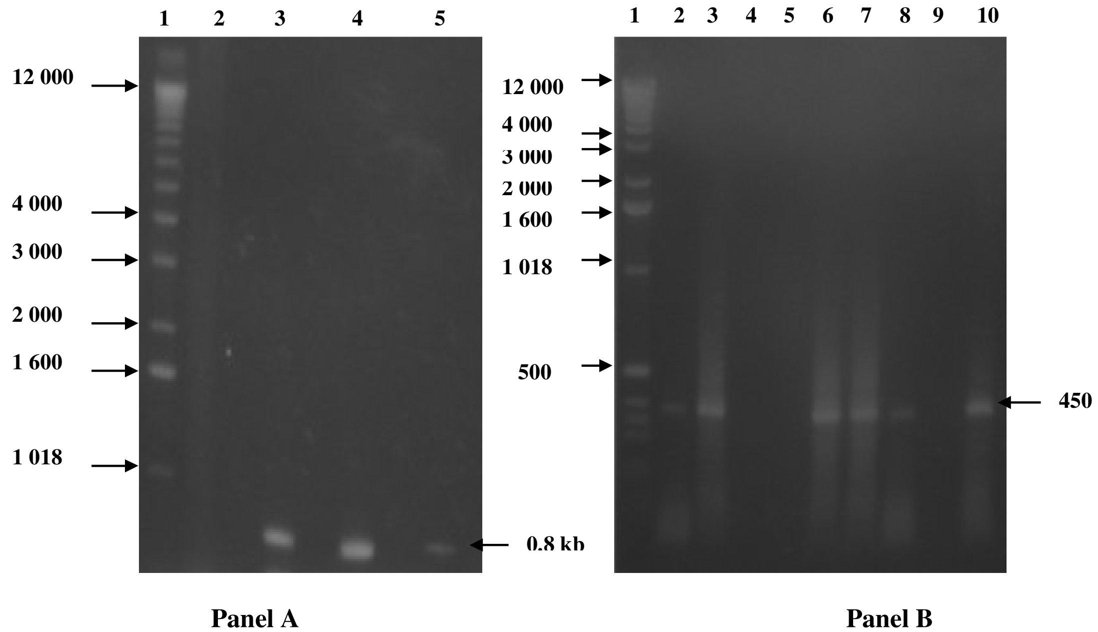

Figure 4. Photograph of 1.0% (w/v) agarose gels of PCR products to detect the presence of transgenes in putatively transformed *N. bethamiana* plants. Panel A: PCR to detect for the presence of the entire CABMV CP gene. The lanes are: 1 – molecular weight marker (1 kb ladder), 2 – negative control, 3 to 5 – putatively transgenic plants. Panel B: PCR to detect for the presence of the core region of CABMV CP gene. The lanes are: 1 – molecular weight marker (1 kb ladder), 2 to 10 – putatively transgenic plants. The CP gene is present in Panel A lanes 3, 4, and 5, and Panel B lanes 3, 6, 7 and 10.

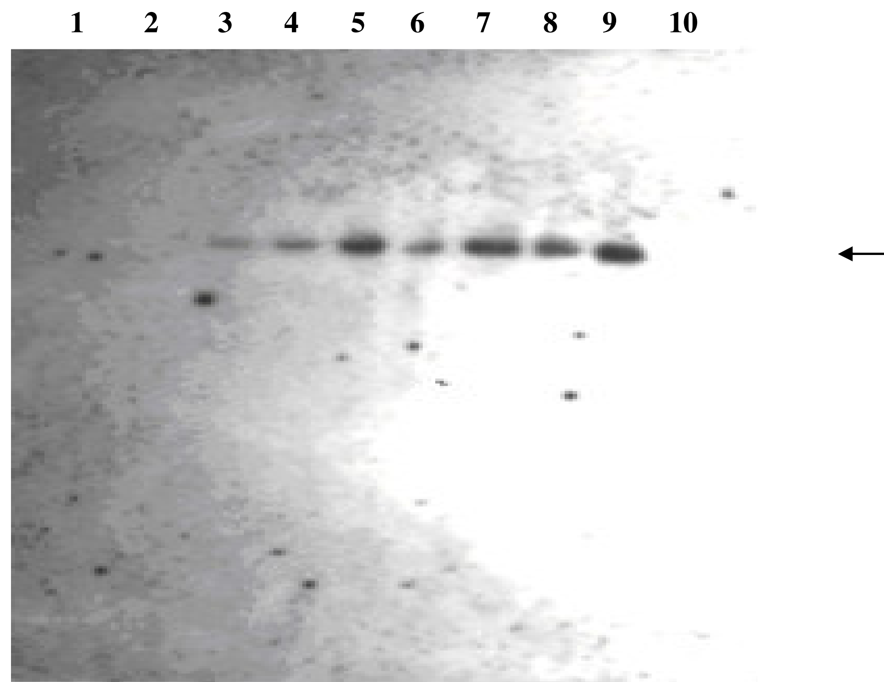

Figure 5. Autoradiogram of a blot of the products of a PCR to amplify the CPcore region of putatively transformed *N. benthamiana* plants. The lanes are: 1 – 1kb ladder, 2 – non-transformed *N. benthamiana*, 3 – line 034, 4 – line 040, 5 – line 043, 6 – line 046, 7 – line 047, 8 – line 048, 9 – line 049, 10 – line 051. The arrow indicates the 450 bp CP_{core} fragment, present in lines 034, 040, 043, 046, 047, 048 and 049.

Table 3. Summary of virus challenge experiments.

Construct	Resistance results				
	Delayed symptom development	Tolerant	Recovery/ New shoots	Modified symptoms	Summary comments
PBI121-CP_k	020-1, 026-3, 026-5, 027-1, 028-17, 035-37, 035-38, 035-39, 035-41, 035-42, 046-5, 046-6, 052-1, 052-2, 052-3, 052-4, 052-5, 052-7, 052-8, 052-9, 061-1, 065-1, 065-2 23 lines	Nil	Nil	Nil	Delayed symptom development. Symptoms obvious on all plants at Day 28. No immunity
PBI121	Nil	Nil	Nil	Nil	No resistance

Plant 028-17 developed a new stunted shoot at 28d. Lines transformed with pBI121 are controls to demonstrate that resistance observed is not due to the presence of the binary vector.

DISCUSSION

Of the 15 putative field isolates of CABMV collected by Sithole-Niang et al. (1996), CABMV-Z3 showed very severe symptoms on cowpea and was the third in the collection. It was thus referred to as CABMV-Z3. The CABMV 3'-terminal region was successfully amplified and cloned into pGEM-T. The cloned amplicon was sequenced and sequence analysis confirmed that it was indeed an isolate of CABMV, CABMV-Z3 as originally isolated and characterized by Sithole-Niang et al. (1996). This experiment was necessary in this study to confirm the identity of CABMV-Z3 and that the virus had not undergone any significant changes due to mutation during passaging experiments.

Sequence comparison in the CP region showed 100% identity. The source of variation from isolate to isolate will become apparent when more sequence information of the different isolates become available.

The amplicon consisted of the CP gene with an ATG codon added upstream. The ATG was added by PCR mutagenesis that also positioned this translation start codon in an optimised context for expression in plants (Lutcke et al., 1987). This construct, pBI121-CP_k, codes for a CABMV-CP that is transcribed and translated into the wild type CABMV CP. The integrity of the clone was verified by sequencing. The binary constructs were used to transform *A. tumefaciens* which were then used in co-cultivation methods to transform *N. benthamiana*.

The binary plasmid DNA yield and quality from *A. tumefaciens* was poor but this was not unexpected since the binary vector is a low copy number plasmid and the common DNA isolation methods are not optimized for *Agrobacterium*. However, this small amount of DNA was used to transform *E. coli* from which good quality DNA was obtained and used to identify intact clones. This verification was necessary since the complete sequence of pBI121 is not available, and deletions and other rearrangements can occur in binary vectors (Frisch et al., 1995). The verified clones were then used in plant transformation experiments.

The number of pBI121-CP_k transformants was high (42 out of 68). Resistance to kanamycin, detection of the CP in an immunodotblot assay and PCR amplification the CABMV CP gene, confirmed as such by both size and hybridization to CABMV CP or CP_{core} probes, showed the transgenic nature of plants. The predominant phenotype was delayed symptom development. Progeny of lines 035, 052, 061 and 065 which had detectable CABMV CP levels are among those which displayed delayed symptom development. The mechanism of resistance in these lines is likely to be CP-MR, with interactions taking place between transgene CP in the transgenic plant and incoming viruses in the challenge inoculums (Bendahmane et al., 2007). These results are consistent with those of Powell-Abel et al. (1986) who showed protection in lines expressing detectable levels of CP. This is also consistent with observations on susceptible lines such as 034 that, even though they are transgenic for CP, did not express detectable levels of CP.

The other lines that display delayed symptom development are progeny of lines 020, 026, 027, 028 and 046 where CABMV CP was not detected by immunodotblot analysis.

It is likely that a mechanism other than CP-MR is at work in these lines, probably RNA-mediated resistance (Doreste et al., 2002; Sivamani et al., 2002). RNA analyses would clarify this possibility. Delayed symptom development is significant resistance because it protects the plant during the early most vulnerable stages of development and protects the plant enough to be able to set seed. Little evolutionary pressure is exerted on the viral pathogen to evolve new strategies to evade protected plants (Simon and Bujarski, 1994). It therefore tends to be longer lasting in the field.

Data from R2 generation of interesting lines is required

because position effects and somaclonal variation makes statistics with first generation transgenic plants unreliable (Nap et al., 1993). Southern hybridisation using DIG chemiluminescent procedure failed to detect the transgenes. This was probably due to low sensitivity of the procedure. More sensitive detection procedure such as the ^{32}P procedure could have detected the transgenes.

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