Characterisation of *Cowpea aphid-borne mosaic virus* (CABMV) and evaluation of pathogen derived resistance to the virus.

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ABSTRACT

Cowpea is a major source of dietary protein in most African traditional diets, and the crop partially replenishes the soil nitrogen content as a result of its association with nitrogen-fixing *Rhizobium* spp, and is used as fodder. However, cowpea suffers from a wide range of production constraints such as viral diseases. Cowpea aphid-borne mosaic potyvirus (CABMV) is one of the most important viral pathogens of cowpea in major cowpea growing regions of the world and significantly reduces cowpea yields of resource poor farmers. In an effort to characterise a CABMV isolate from Zimbabwe, designated CABMV-Z3, using classical virology techniques the host range of CAMBV-Z3 was tested. The symptoms produced on susceptible *Nicotiana bethamiana*, *N. clevelandii*, *Vigna unguiculata*, *Chenopodium amaranticolor* and *Ch. quinoa* plants were described. *Nicotiana glutinosa*, *N. tabacum*, *N. rustica*, *Phaseolus vulgaris* and *Cucumeris sativus* were shown to be non-hosts of the virus. The CABMV-Z3 was purified for the first time and polyclonal antiserum was produced against the virus. Full-length cDNA clones of CABMV-Z3 were made by long-range reverse transcription-polymerase chain reaction (RT-PCR).

Another aspect of the study was to determine the most effective virus resistance mechanism using the CABMV-Z3 coat protein (CP) gene, in tobacco as a model host since no reliable cowpea transformation protocol was available at the time. The 1.2 kb 3' terminal region of CABMV-Z3 was amplified using RT-PCR technique and cloned into a PCR cloning vector pGEM-T, and named pGEM-CPRep. This clone was used as template in a PCR to amplify a CP gene optimised for expression in plants (CP_k), an untranslatable CP gene with stop codons in all three reading frames (CP_{stop}), an anti-sense CP (PC), and the central region of the CP gene (CP_{core}). All four amplification products were provided with promoters and terminators from expression cassette vector pCa2Nos, and ligated into the unique *Hin*d III site of the binary plasmid pBI121, to result in plasmids pBI121-CP_k, pBI121-CP_{stop}, pBI121-PC and pBI121-CP_{core}. The constructs were used in *Agrobacterium*-mediated transformation of *Nicotiana benthamiana* leaf sections following the co-cultivation method. Regenerated plants were analyzed by PCR and Southern hybidization. R1 seedlings were assayed for kanamycin resistance and for presence of CP, and challenged with CAMBV-infected sap. Lines showing delayed symptom development, tolerance and recovery were identified but no line showing immunity was identified. This was 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.

The ultimate goal of the project was to produce cowpea lines that are resistant to CABMV. However, there was no efficient, reliable and reproducible cowpea transformation and regeneration system. A cowpea transformation protocol was developed and evaluated, utilizing the binary constructs specified above. The binary constructs were electrophoresed into the apical meristems of developing cowpea seedlings under various conditions including different voltage and current settings as well as pretreatment of seedlings with acid or plant growth regulators. Preliminary screening was done using GUS assays, PCR and Southern analysis, and the plants allowed to set seed. The T1 seeds were germinated *in-vitro* on MS plates. The results show that DNA delivery to meristematic cells was successful, leading to transgenic sectors of the plants. However, the chimeric nature of the plants poses a problem, as there is need for a reliable non-destructive mechanism to track the transgenic branches of the chimeras. This transformation method has the potential to avert some of the concerns often raised against transgenic plants since it does not necessarily require selectable marker and reporter genes, and avoids the expensive and laborious tissue culture step.

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1 INTRODUCTION

Cowpea (*Vigna unguiculata*) is a leguminous crop grown by many resource poor farmers in the developing world. The crop is of social, economic and dietary importance. In many traditional African societies, cowpea is grown by women who use it as a strategic food source, and a security 'fall-back' crop in seasons of crop failure. Some societies use cowpea as a currency of barter trade. The nutritional value of cowpea is high, with dried grain containing up to 24% protein and 56% carbohydrate (Madamba, 1997). The seed proteins are deficient in tryptophan and the sulphur-containing amino acids methionine and cysteine, but still complement in part the protein deficiency of maize and cassava, the staple for most of Africa (Bressan, 1985).

As in other leguminous plants, *Rhizobium* in symbiosis with cowpea can fix up to 32 kg N /ha, replenishing the soil nitrogen in a sustainable manner (Shumba, 1990). This is of importance when the crop is grown intercropped with some other main crop, usually maize, or as a monocrop as is done with the newer higher yielding varieties. Cowpea is also an important fodder crop.

The major constraints in cowpea production are low yields of traditional varieties, and susceptibility to fungal and viral pathogens and insect damage. Efforts are underway in laboratories around the world to breed cowpea varieties with pest and disease resistance, and increased yield. One aspect that has not been adequately covered is that of virus resistance. One of the most important viruses of cowpea is *Cowpea aphid-borne mosaic potyvirus* (CABMV). CABMV belongs to the *Potyviridae* family and infects cowpea resulting in high yield reductions, with the severest of 18 - 87% crop loss reported in Iran, and a probable 100% loss reported in Nigeria (Thottappilly and Rossel, 1992). Our particular interest is in genetically engineering resistance to CABMV.

Viruses can be studied using classical techniques of mechanical passaging to potential hosts to determine possible hosts, and also isolation, purification and immunological evaluation. With the advent of recombinant DNA technology, whole viral genomes or parts thereof can be amplified using techniques such as reverse transcription (RT), polymerase chain reaction (PCR), cloning and expression. Recombinant DNA technology has also resulted in the development of new approaches to the development of virus-resistant plants, such as those included in pathogen-derived resistance (PDR), (Wilson, 1993).

The aim of this study was to evaluate PDR to CABMV. The specific objectives of this research project were to develop tools for CABMV monitoring and study, and evaluate coat protein-mediated resistence (CP-MR), sense and antisense RNA-mediated resistance to CABMV in tobacco, and further develop this in cowpea. While breeding for virus resistance is an ongoing activity in various laboratories, truly resistant germplasm is unavailable. Pathogen-derived resistance to viruses 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. In this study, the host range of an isolate of CABMV from Zimbabwe, CAMBV-Z3 was tested, and the symptoms produced on the susceptible plants were described. The CABMV-Z3 was purified for the first time and polyclonal antiserum was produced against the virus, and full-length clones of CABMV-Z3 were also made. The polyclonal antiserum against the virus and full-length clones are important tools in characterizing a virus and its infection cycle. Pathogen-derived resistance to CABMV was evaluated. This was achieved through making various CABMV CP constructs that were then used to transform *Nicotiana benthamiana*, and evaluated for resistance to CABMV. A cowpea transformation protocol based on electrophoresis of DNA into the apical meristem of cowpea seedlings was also developed and evaluated, utilizing the CABMV-Z3 CP binary constructs.

2 LITERATURE REVIEW

2.1 Cowpea crop and its production constraints

2.1.1 The cowpea crop

Cowpea is the common name for *Vigna unguiculata* (L.) Walp., synonym *Vigna sinensis* (L.) Savi ex Hassk, section *Catiang*, also known as southern pea or blackeye pea, and is one of the seven cultivated species in the genus *Vigna* (Savi) Verdc. (Ng, 1992). The other members of the genus are mung bean, *V. radiata* (L.) R. Wilizek; black gram, *V. mungo* (L.) Hepper; rice bean, *V. umbellate* (Thunb.) Ohwi and Ohasi; adzuki bean, *V. angularis* (Wild) Ohwi and Ohasi; moth bean, *V. aconitifolia* (Jacq.) M.M. and S. all of which are in subgenus *Ceratotropis*, and bambara groundnut, *V. subterranean* (L.) Verdc., in section *Vigna*. Members of subgenus *Ceratotropis* originated in Asia, while *V. unguiculata* and *V. subterranean* originated in Africa. Intraspecific crosses between *V. unguiculata* ssp *unguiculata* and other members of the species are fairly easy and show a lot of potential for improving cultivated cowpea, but inter-specific crosses are very difficult to make (Ng, 1992).

The cowpea centre of diversity is in Savannah regions of West Africa, including parts of Nigeria, Niger, Burkina Faso, Benin and Togo. There are some wild cowpea centres of diversity in southeastern Africa as well (Ng & Marechal, 1985).

Although cowpea lacks methionine, cysteine and tryptophan, it forms an important source of essential amino acids in many tropical diets that predominantly consist of starchy cereals, roots and tubers (Bressan 1985). In fact, the cereals complement the sulphur deficiency of cowpea (Duranti & Gius, 1997).

2.1.2 Cowpea improvement

Cowpea is a highly self-pollinating plant although significant levels of out-crossing have been detected in the wild (Ng & Marechal, 1985). Studies suggest that gene exchange between cowpea and its wild relatives occurs naturally in the wild. The Grain Legume Improvement Program (GLIP) of the International Institute of Tropical Agriculture (IITA) has collected a large number of native cowpea cultivars (Ng & Marechal, 1985). The reason why the number of native cultivars is so large is that each variety is adapted to local conditions such as drought and low soil fertility, and has retained its culinary traits, for which it is prized.

Crop improvement efforts funded by western governments and companies in the 20th century did not include cowpea and other crops of importance to the developing world because the crops were not considered commercial cash crops and had little promise for return on investment in research. After identifying the important role of cowpea in the socio-economics of developing countries, the donor community recently started funding development of improved cowpea lines. The main reason for the change in funding policy was the realisation that cowpea was the main protein source for the resource poor subsistence farmers, and also contributed to soil fertility. The first major effort was in the establishment of a cowpea breeding program within the IITA. In 1989, a unit dedicated to cowpea transformation was also established at IITA, after the realisation that conventional breeding alone would not incorporate all the desired traits into cowpea. More recent efforts to improve cowpea include the establishment of the Network for the Genetic Improvement of Cowpea for Africa (NGICA) and African Agricultural Technology Foundation (AATF), (http://www.entm.purdue.edu/ngica/reports/SGM-Nairobi.pdf).

Use of genetically modified (GM) crops is normally considered when conventional breeding cannot yield a solution, for example, if the trait is not in germplasm, if the goal is enhanced

nutritional quality, or for specialty products such as vaccines. Genetically modified crops will also be supported when the benefits of the technology outweigh the cost and when intellectual property rights and regulatory issues can be addressed.

2.1.3 Constraints to cowpea production

Even though yield potentials of 1.5 – 3 t/ha have been reported when cowpea is grown traditionally inter-cropped with maize, the average yield of traditional cowpea varieties is 200 - 300 kg/ha or less (Jackai *et al*., 1985). Dube (1995) reported that in monocrop, yields of the traditional varieties averaged 335 kg /ha while the newer improved varieties such as CBC1 yielded 679 kg/ha. These high yields could also be obtained in intercrops with maize provided appropriate fertilizers were applied. Most farmers do not find these yields attractive, and so cowpea remains a poor man's crop. The main reasons why cowpea fails to realise its yield potential are the poor soils and numerous pathogens. The major pathogens of cowpea in Africa are listed in Tables 1 and 2 overleaf.

Table 1. List of the major pathogens of cowpea in Africa: insect pests and the stage of production where the particular insects have the most damaging effect (Singh & Jackai, 1985).

Insect Pests		
Order	**Species**	**Stage of cowpea production**
Homoptera: aphids (Aphididae)	*Aphis craccivora* Koch and *A. fabae*	Homoptera are important sap-sucking, leaf eating and virus transmitting insects.
leafhoppers (Cicadellidae)	*Empoasca dolichi* Paoli, *E. signata* Haust (Singh & Jackai, 1985)	
Hemiptera: pod bugs – Coreidae	*Clavigralla tomentosicollis* Stal. (synonym: *Acanthomia tomentosicollis* Stal.), *C. shadabi* Dolling (synonym: *A. horrida* Germar) and *C. elongata* Signoret and *Anoplocnemis curvipes* Fabricus	Feed on pods
Alydidae	Alydidae – *Riptortus* spp, especially *R. dentipes*	
Pentatomidae	Pentatomidae – *Nezara viridula* Linnaeus, *Aspavia armigera* Fabricius	
Thysanoptera: Thripidae	legume bug thrips, *Megalurothrips sjostedti* Trybom (synonym: *Taeniothrips sjostedti* Trybom); foliage thrips, *Sericothrips occipitalis* Hood.	Foliage
Diptera: Agromyzidae	beanfly, *Ophiomyia phaseoli* Tryon (synonym: *Melanagromyza phaseoli* Tryon)	
Lepidotera: Pod borers: Pyralidae	legume pod borer, *Maruca testulalis* Geyer; *Etiella zinckenella* Treitschke	Bores into pods
Nocuidae	African bollworm, *Heliothis armigera* Hubner	
Tortricidae	cowpea seed moth, *Cydia ptychora* Meyrick (synonym: *Laspeyresia ptychora* Meyrick	
Foliage feeders: Noctuidae	Egyptian leaf worm, *Spodoptera littoralis* Boisdural (synonym: *Prodenia litura* Fabricius)	Feed on leaves and also bores or feed into pods
Coleoptera: Beetles:- Chrysomelidae	cowpea leaf beetle, *Ootheca mutabilis* Shalberg; *O. bennigseni* Weise, stripped foliage beetle, *Medythia quaterna* Fairmaire (synonym: *Luperodes lineata* Kars	The beetles feed on foliage and flowers, and also act as vectors for viruses
Meloidae	Blister beetles, *Mylabris* spp., and *Coryna* spp.;	
Lagriidae	*Lagria villosa* Fabricius, *Chrysolagria* spp	
Weevils:- Cucurlionidae	stripped bean weevil, *Alcidodes leucogrammus* Erichson	Adults feed on leaves, lay eggs on stem, larvae tunnel and feed inside the stem.
	pod weevil, *Piezotrachelus varius* Wagner (synonym: *Apion varius* Wagner),	Lay eggs on green pods, larvae feed and pupate inside the seed; tiny black weevils emerge from dry pods.
Bruchids – Bruchidae	storage weevils – *Callosobruchus* spp., especially *C. maculates* Fabricius and *C. chinensis* Linnaeus	Infest pods when almost mature and multiply in stored seeds, causing up to 30% weight loss in 6 months.

Table 2. List of the major pathogens of cowpea in Africa: fungal and bacterial diseases (Emechebe & Shoyinka 1985), viruses (Thottappilly & Rossel, 1992), parasitic weeds and nematodes (Emechebe & Shoyinka 1985).

Fungal and Bacterial Diseases	
Disease	**Causative agent**
Anthracnose	*Colletotricum lindemuthianum*
Ascochyta blight	*Aschochyta phaseolorum*
Brown blotch	*Colletotrichum* spp especially *C. capsici*, occasionally *C. truncatum*
Brown rust	*Uromyces appendiculatus*
'Cercospora' leaf spot	*Pseudocercospora cruenta* and *Cercospora canescens*
Macrophomina charcoal rot and ashy stem blight	*Macrophomina phaseolina* (sclerotial stage is *Rhizoctonia bataticola*)
Phytophthora stem rot	*Phytophthora vignae*
Pythium soft stem rot	*Pythium aphanidermatum*
Seedling decay and seedling damping-off complex	*Pythium aphanidertum*, *Colletotricum capsici*, *Rhizoctonia solani* (teliomorph is *Thanatephorus cucumeris*) and *Macrophomina phaseolina* (sclerotial stage is *Rhizoctonia bataticola*)
Septorial leaf spot	*Septoria*
Sphaceloma scab	*Sphaceloma*
Web blight	*R. solani*
Wilts	*Fusarium* spp, *Schlerotium rolfsii*
Yellow blister (false rust)	*Synchytrium dolichi*
Bacterial blight and canker	*Xanthomonas campestris* pv vignicola
Bacterial postule	*Xanthomonas* spp.
Viral Diseases	
Virus	**Vector**
Cowpea yellow mosaic virus (CYMV)	Beetle
Cowpea mottle virus (CMoV)	Beetle
Southern bean mosaic virus (SBMV)	Beetle
Cowpea aphid-borne mosaic virus (CABMV)	Aphid
Cucumber mosaic virus (CMV)	Aphid
Cowpea golden mosaic virus (CGMV)	White fly
Cowpea mild mottle virus (CMMV)	White fly
Sunn-hemp mosaic virus (SHMV)	Unknown
Parasitic Weeds	
Striga gesneriodes	
Actra vogeli	
Nematode Diseases	
Root knot nematode	

The economic impact of the diseases can be reduced by various approaches including use of resistant varieties, chemical control, good cultural practices and integrated disease management. Natural resistance to most insects is not widely available in cowpea. Therefore attempts to introduce resistance were by genetically engineering cowpea to produce the *Bacillus thuringiensis* (Bt) endotoxin proteins (Murdock, 2001; http//www.isp.msu.edu/crsp/WorkplansforFY05/wa5-a1.pdf). Protease inhibitors, amylase inhibitors and lectins could also be used against these insect pests (ibid). If an efficient transformation and regeneration protocol were to become available, this is one area that would greatly benefit.

Cowpea lines resistant to parasitic weeds have been identified and are being utilized in breeding programmes (Boukar *et al*., 2004). However, the presence of numerous strains of the parasites complicates the programme. More recently, a breeding line, IT93K-693-2, was shown to have resistance to all known *S. gesnerioides* races. Utilizing information from the cowpea genetic linkage map (Ouedraogo *et al*., 2002), a sequence characterized amplifiable region (SCAR) marker for the resistance has been cloned for use in marker-assisted breeding programs (Boukar *et al.*, 2004).

An understanding of viruses of cowpea and the diseases they cause is important if cowpea is to be produced economically. Precise identification of the parasitic viruses is essential for effective control and definition of geographical distribution (Taiwo *et al*., 1982). Cowpea aphid-borne mosaic virus (CABMV) and cowpea mottle virus (CMoV) are the most important cowpea viruses in Africa on the basis of geographical distribution, pathogenic variation and yield loss. The CMoV is easily distinguished from CABMV since CMoV has a bipartite RNA genome, is isometric, 25 nm in diameter and is beetle transmitted. The CABMV, a potyvirus, has a single positive sense RNA genome enclosed into a flexuous filamentous particle by capsid protein

monomers. The symptoms of CABMV infected cowpea plants are a severe mosaic, with severity depending on cowpea cultivar and virus strain. Dark green vein banding, leaf distortion, blistering and stunting also occur. The virus is transmitted by sap inoculation and by aphids in a stylet-borne non-persistent manner. These aphid vectors include *Aphis craccivoria*, *A. gossypi*, *A. spiraecola*, *A. mediccaginis*, *Macrosiphum euphorbiae*, *Myzus persicae*, *Rhopalosiphum maidis* and *Cercatophis palmae* (Thottappilly & Rossel, 1992). Non-feeding aphids can retain infectivity for up to 15 hours after virus acquisition (Bock & Conti, 1974). The other common aphid-transmitted virus that can infect cowpea is cucumber mosaic cucumovirus (CMV). The CMV can be distinguished from CABMV in that it can be sap-inoculated into *N. glutinosa* whereas CABMV cannot be sap-inoculated into *N. glutinosa* (Thottappilly & Rossel, 1992).

2.1.4 Cowpea aphid-borne mosaic virus

Cowpea aphid-borne mosaic virus (CABMV) is a member of the *Potyviridae* family of viruses, whose infections are characterized by pin-wheel inclusions (Bock & Conti, 1974; 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).

Cowpea aphid-borne masaic virus was first reported in Italy, and then in Australia, East Africa, India, Indonesia, Iran, Japan, Morocco, the Netherlands, Papau New Guinea and Sri Lanka (McKern *et al*., 1994). The original isolates of CABMV were not available for direct comparison, and so proper identification was not possible, and some isolates originally thought to be CABMV turned out to be black eye cowpea mosaic virus (BlCMV). The more definitive tests that resulted in the rectification of this misidentification were resistance/susceptibility tests of selected cowpea lines to either BlCMV or CABMV (Taiwo *et al*., 1982). It was determined that

the Moroccan and Cypriot isolates of CABMV were identical, and the Kenyan and Nigerian isolates of CABMV were different from these two, but similar to the Florida and New York isolates of BlCMV. These results are in agreement with the results of Taiwo & Gonsalves (1982) who obtained antisera against single lesions of each of the isolates used by Taiwo and co-workers, and then used enzyme-linked immunosorbant assay (ELISA) and immunodiffusion in sodium dodecyl sulphate to compare the isolates (Taiwo *et al*., 1982).

CABMV and South African passiflora virus (SAPV) were shown to be similar to the Moroccan strain of CABMV, but distinct from bean common mosaic virus (BCMV) and bean necrosis mosaic virus (BCNMV), using HPLC profiles of tryptic peptides and partial amino acid sequence analysis (McKern *et al*., 1994). It was therefore suggested that SAPV is a strain of CABMV and should be renamed CABMV-SAP (ibid).

The host range of CABMV includes Leguminosae although most strains also infect members of Amarathaceae, Labiatae and Solanaceae (Bock & Conti, 1974). Seed transmission is slight. Thermal inactivation of the virus occurs at 57 – 60°C, the sap dilution end-point is between 10^{-3} and 10^{-4}. The virus can be stored at –20°C for up to 3 days without losing infectivity. Frozen infected leaves retain infectivity up to 7 weeks (Bock & Conti, 1974). The unaggregated virus particles have a sedimentation coefficient of 150 S.

CABMV induces numerous granular inclusions in epidermal cells of cowpea, but few in petunia. The virus infects almost all parts of cowpea plants, including anthers, ovaries and embryos (Bock & Conti, 1974). The occurrence of granular inclusions in infected cells is characteristic of all potyvirus infections.

2.2 Potyvirus molecular biology

2.2.1 Properties of the *Potyviridae* family

Dougherty & Carrington (1988) reviewed the molecular biology of potyviruses, the largest group of viral pathogens with over 200 members. Potyviral genomes are uni-component with a single-stranded positive sense infectious RNA of about 10 kb with a 5' VPg and a 3' polyA tail, and is transcribed into a polyprotein of about 350 kDa (Dougherty & Carrington 1988, Urcuqui-Inchima *et al*., 2001).

The 2 000 capsid protein units of each virion are of one type, each being 30 – 45 kDa and showing 60 – 80% amino acid similarity within the group (Dougherty & Carrington 1988). The VPg, a virus-encoded protein attached to the 5' terminus of the genomic RNA is probably involved in viral replication. The VPg has been implicated in the initiation of RNA synthesis, and also probably plays a role in aphid transmission (Mlotshwa et al., 2002). The size of the VPg varies greatly, being 6 kDa in *Tobacco etch potyvirus* (TEV) while that of *Tobacco vein mottling potyvirus* (TVMV) is 24 kDa.

All potyvirus–infected plants have cytoplasmic inclusion proteins, made up of 65 – 70 kDa monomers (Dougherty & Carrington 1988). They associate with plasma membrane and aggregate to form 'spikes' which elongate and eventually dissociate from the membrane. The morphologies of the scroll-like or pinwheel shaped inclusions bodies are virus-specific, and have been suggested as taxonomic and diagnostic features. These inclusion bodies are analogous to the 2PC proteins of picornaviruses, and are therefore likely to be involved in cell-to-cell movement and replication.

Cytoplasmic amorphous inclusion (CI) proteins (Fig 1) have been detected only in some potyvirus infections. In pepper mottle virus (PeMV), the 51 kDa protein which has been shown by biochemical and immunological evidence to be the helper component - protease (HC-Pro) for insect transmission, is the major component of the CI protein. The cytoplasmic amorphous inclusion proteins are probably inactive or unprocessed forms of the HC-pro since antiserum raised against these proteins do not inhibit aphid transmission.

Nuclear inclusion (NI) proteins (Fig 1) are made up of 2 virally-encoded components that are found in all potyviruses but only aggregate to form stable structures in a few viruses. In TEV, the two components are the 58 kDa protein (NIa) which is an RNA-dependent RNA polymerase (RdRp, viral replicase) and the 49 kDa protein (NIb) which is a viral-encoded proteinase responsible for cleavage of the polyprotein.

2.2.2 Genomic organisation of CABMV

A large number of potyviral genomes have been fully sequenced, including TEV (Allison *et al*., 1986), TVMV, *Potato Y virus* (PVY), *Plum pox potyvirus* (PPV) (Reichmann *et al*., 1992), and more recently CABMV (Mlotshwa *et al*., 2002). The genomes of the Johnson grass strain of sugarcane mosaic virus (SMV) (Gouch *et al*., 1987) and pepper mottle virus (Dougherty *et al*., 1985) have been partially sequenced. All potyviral proteins described are cleaved from a single polyprotein translated from the genomic RNA. The organisation of the genes coding for these proteins on the TEV and TVMV genomes have been determined by cell-free translation systems, analysis of potyviral RNA and nucleotide sequencing (Hellmann *et al*., 1986). Analysis of all the available sequences shows that potyviral cistronic organisation is basically similar, with only minor differences.

The complete genomic sequence of a CABMV isolate from Zimbabwe was determined (Mlotshwa *et al*., 2002) and is used here as an example. It consists of 9 465 nucleotides excluding the poly-A tail, and 9 159 of these nucleotides are coding, and translate to a polyprotein of 3 053 amino acids. The genomic organisation is as illustrated below.

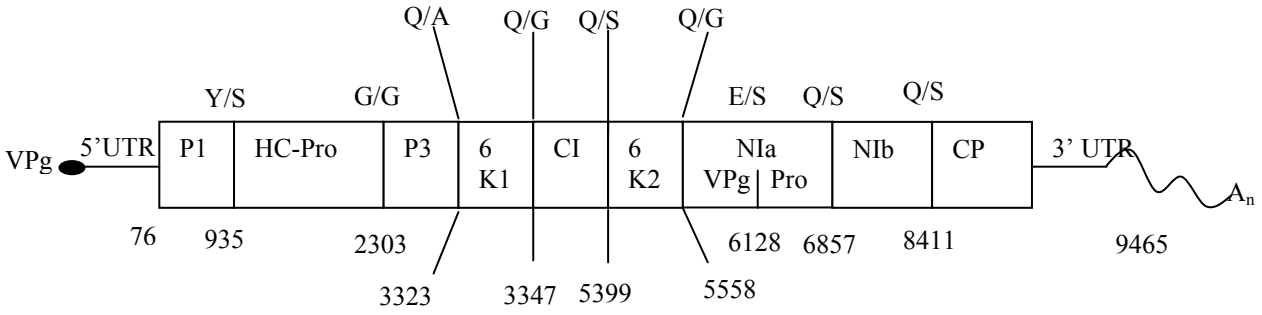

Figure 1: Illustration of the genomic organisation of CABMV. The autocatalytic cleavage sites are shown above, and the corresponding nucleotide sequence position is numbered below the diagram. VPg – genome-linked virus-encoded protein; P1 – first protein; HC-Pro – host component – proteinase; P3 – third protein; CI – cytoplasmic inclusion protein; NIa – nuclear inclusion protein a; Pro – proteinase; NIb – nuclear inclusion protein b, i.e. replicase; CP – coat protein. Adapted from Mlotshwa *et al*., (2002).

The first protein, P1, is involved in polyprotein processing as it autocatalytically cleaves itself at its C terminus (Carrington *et al*, 1989, Verchot *et al*., 1991). The P1 is also involved in genome amplification and was once speculated to be involved in cell-to-cell movement. The second protein, HC-Pro is also involved in polyprotein processing by autocatalytically cleaving its C terminus (Carrington *et al*, 1989, Verchot *et al*., 1991). The HC-Pro is also involved in aphid-mediated transmission, genome amplification, cell-to-cell and long distance movement, trans-activation of replication of heterologous viruses, and suppression of post-transcriptional gene silencing, PTGS (Mlotshwa *et al*., 2002).

The third protein, P3, is a cofactor in polyprotein processing, and the 6K1 is involved in genome amplification. The cytoplasmic inclusion (CI) protein has been proposed to be a helicase with ATPase activity, causing unwinding of double-stranded RNA in a 3' to 5' direction as shown in PPV (Reichmann *et al*., 1992). By analogy to picornaviruses (e.g. foot and mouth disease virus, poliovirus), potyviruses are expected to replicate in the cytoplasm in a membrane-associated process. The CI is therefore involved in the attachment of replication complexes to host cell membranes. The CI protein is also involved in cell-to-cell movement. Excess CI protein accumulates in the cytoplasm where they form the cylindrical bodies that characterise all potyviral infections. The second 6K protein, 6K2 is involved in genome amplification and intracellular transport.

A viral encoded proteinase (NIa), 49 kDa in TEV, autocatalytically cleaves the polyprotein. The conserved Cys-(13 – 17 Xaa)- His sequence is the proposed active site. The free thiol group residue is utilized as a nucleophile during peptide bond cleavage, and the His residue is a general acid/general base. The cleavage site for the viral encoded proteinase is:

Glu-Xaa-Xaa-Tyr-Xaa-Gln-Ser or Gly,

where Xaa is a neutral or hydrophilic amino acid, and the Gln-Ser or Gly bond is the sessile bond. The first nuclear inclusion (NIa) protein has a protease domain that is involved in polyprotein processing. The NIa protease autocatalytically cleaves itself from the polyprotein at both the N and C termini and also recognises and cleaves the rest of the cleavage sites shown in Figure 1 (Carrington & Dougherty, 1987). The other domain of the NIa is the VPg domain that primes genome replication and has a role in subcellular transport and a host genotype-specific long distance movement factor.

The second nuclear inclusion (NIb) protein is the replicase, an RNA-dependent RNA polymerase (RdRp). The RdRp is found in all positive strand RNA viruses and has a conserved motif of about 300 amino acids (Dolja & Carrington, 1992). Motifs V and VI that contain the highly conserved Gly-Asp-Asp (GDD) sequence have been suggested to be the nucleotide triphosphate binding site. The other motifs are thought to be for RNA docking, and the RdRp has no proof-reading ability. Potyviral genomes also have 5' and 3' terminal non-coding regions and intersignal sequences. The positive strand RNA viruses are proposed to have evolved by point mutations and recombination by the copy-choice mechanism (Dolja & Carrington, 1992). Excess NIa and NIb proteins accumulate in the nucleus to form nuclear inclusion bodies.

The coat protein (CP) encapsidates the genomic RNA and is essential for aphid-mediated transmission, cell-to-cell and long distance movement (Shukla *et al*., 1994). The (ileu/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).

2.3 Recombinant DNA technology

Recombinant DNA techniques have enabled the field of molecular virology to grow and occupy a key position in virology. These techniques include polymerase chain reaction (PCR), reverse transcriptase-PCR (RT-PCR) and gene cloning, and are often used in conjunction with other techniques such as molecular markers and production of monoclonal and polyclonal antibodies (Hull 2002). The RT-PCR technique allows cloning of segments of the RNA viruses even before the virus can be purified. Primers can be designed on the basis of conserved sequences, identified by comparing sequences of related viruses that have been sequenced. An important feature of potyviruses is the poly-A tail at the 3'-end of the genome (Reichmann *et al*., 1992). An oligo dT primer can therefore be used in reverse transcription or cDNA synthesis, leading to

amplification and cloning of the 3'-end of the genome. The coat protein gene is at the 3' end of the virus, and its sequences have also been used in the identification and classification of potyviruses.

If the cloned viral sequences are to be expressed in plants, there is a need to add certain sequence elements that are required for efficient expression in plants. These modifications include changes to the untranslated regions since differential translation occurs through interactions involving 5' untranslated sequences of mRNA. *In vitro* experiments have shown that mutating the nucleotide at position –3 from the A of the AUG translation start codon retards the translation rate, but to different extents in wheat germ and reticulocyte systems (Lutcke *et al*., 1987). A survey of plant and animal sequences revealed that the consensus for animals is CACC AUG and that for plants is AACA AUG (Lutcke, *et al*., 1987). In experiments to construct recombinant binary vector plasmids for use in plant transformation, the appropriate consensus sequences must therefore be introduced at the correct position between the promoter and the AUG translation start codon.

After plant transformation there is need for techniques that demonstrate the presence of transgene in transformed plants. A widely used sensitive method for detection of DNA immobilized on solid support such as nitrocellulose or nylon membranes is hybridization to radioactively labeled (^{32}P) probes. However, this method is associated with problems of handling and disposal of radioactive material. Digoxigenin (DIG) offers a good alternative since it is non-radioactive and can be very sensitive. DIG is a steroid hapten that can be coupled to at least one of the nucleotides used in the synthesis of DNA, RNA or oligo nucleotide probes, thereby effectively labelling them with DIG (Kessler *et al*., 1990). After hybridization, the DIG labeled probes are reacted with anti-digoxigenin Fab fragments conjugated to alkaline phosphatase, and then visualized with colorimetric substrates such as nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl-

phosphate (NBT/BCIP), or with chemiluminescent substrates like CSPD or CDP-star (Roche Molecular Diagnostics, Germany). Enzymatic dephosphorylation of the chemiluminescent substrate leads to light emission, which can then be recorded on Polaroid or X-ray film.

2.4.0 Virus characterisation

Following discovery of a putatively new virus, the virus must be characterized in detail in order to determine its relationship to other known viruses, for example whether it is a strain of a known virus, a new species or genus. Virus characterisation includes determination of the host range of the virus, description of symptoms of infection in the various hosts, purification of the virus and description of the virus under the electron microscope, including determination of its dimensions. Once purified, the virus can be used to produce polyclonal antiserum against the virus. More recently, viruses are also characterised by sequencing their genomes, determination of how the genomes are expressed, and determination of the functions of the various nucleic acids and proteins encoded for by the genome. With this understanding, infectious transcripts can be made, and used in more controlled experiments to study the virus, transportation and longer-term storage. Virus purification, production of polyclonal antisera and infectious transcripts are considered in more detail below.

2.4.1 Virus purification

An important step towards the full understanding of any virus is its purification. Testing of Koch's postulates, host range, symptoms and many other attributes of the virus can then be done. This is especially important for RNA viruses such as potyviruses where, with the purified virus, it is easier to obtain viral RNA that serves as the best template for cDNA synthesis and reverse transcription-polymerase chain reactions (RT-PCR).

To purify a plant virus, the virus-infected plant material must be macerated and homogenized or ground to break the cells habouring the virus, in the presence of buffers and stabilizing substances that prevent virus inactivation (Bos, 1983). The virus must then be separated from the cell debris by filtration and low speed centrifugation. Organic solvents are often added to the macerate to release the virus from membranes or cell organelles and to clarify the suspension (Bos, 1983). Host proteins can be coagulated by freezing the plant material before extraction or by heating the extract for 10 min at 50 – 60˚C. Coagulated material and coarse cell debris are removed by filtration through cheesecloth or by low speed centrifugation. Further purification and concentration of virus can be achieved through filtration using Chamberland filters or gel filtration; precipitation with ammonium sulphate or hydrophilic organic compounds such as ethanol, acetone, or polyethylene glycol (PEG); or adsorptions on calcium phosphate gel, charcoal, bentonite clay, or diethylamino-ethylcellulose; electrophoresis; or centrifugation (Bos, 1983).

2.4.2 Production of polyclonal antisera

When whole or partially degraded purified virus particles are injected into an animal, the viral particles act as antigens and provoke an immune response against the antigens which results in the production of antibodies against the virus (Bos, 1983). Of particular interest is the immunoglobulin IgG, found in cleared blood serum of the organism. The antiserum is polyclonal in nature because the antigens present a variety of surfaces, each set of which results in an IgG molecule with a particular 3-dimensional structure. Monoclonal antiserum with only one species of IgG molecules can be produced by isolating and propagating individual IgG molecules.

The reaction between virus and antibodies in antiserum can be visualised and quantified by various techniques broadly classified into direct methods such as precipitation tests, diffusion

tests and agglutination tests, and indirect methods such as neutralisation of infectivity, complement-fixation test and EIA/ELISA.

2.4.3 Infectious transcripts

Potyviruses are positive sense RNA viruses, and therefore have RNA genomes that can act directly as mRNA in host cells. The genomic RNA can be used as template to reverse- transcribe the RNA to DNA. The DNA can then be used in an *in vitro* transcription reaction to make many copies of the viral genomes that are infectious if delivered to host cells. Infectious transcripts have been made for TEV **(**Allison *et al.*, 1986), *Zucchini yellow mosaic potyvirus* (ZYMV; Lin *et al*., 2002) and *Clitorial yellow vein tymovirus* (CYVV; Uyeda and Takahashi 1997).

Among potyviruses, transcripts have been synthesized *in vitro* from full-length cDNA clones with bacterial phage promoters and proven to be infectious for PPV (Riechmann *et al*., 1990), ZYMV (Gal-On *et al*., 1991), TEV (Dolja *et al.,* 1992), *Pea seed-borne mosaic potyvirus* (PSbMV; Johansen *et al*., 1996), *Peanut stripe potyvirus* (PStV; Flasinski *et al*., 1996), *Potato A potyvirus* (PVA; Puurand *et al*., 1996), TVMV (Domier *et al.,* 1989; Nicolas *et al.,* 1996), PVY (Jakab *et al*., 1997), *Turnip mosaic potyvirus* (TuMV; Sanchez *et al*., 1998) and *Papaya ringspot potyvirus* (PRSV) (Chiang & Yeh, 1997).

2.5 Approaches to virus resistance

2.5.1 General approaches

Disease resistant crops can be obtained through classical breeding or by genetic engineering. In classical breeding, an existing cultivar with natural resistance to the disease is identified and then crossed with the cultivar to be improved. The disadvantages of this approach include the following: (i) the plant has to cross with a compatible disease resistant species to produce viable

progeny; (ii) other important traits of the cultivar to be improved may be lost as a result of the cross; (iii) the whole process takes a very long time, and is labour-intensive and expensive. Genetic engineering for disease resistance results in the addition of only a single trait of interest to a cultivar already selected for its existing advantages, which are retained in a selfing species like cowpea. Since its inception, there has been worries of real, potential and imagined risks associated with genetic engineering, but objective case by case evaluation shows that the real and potential benefits of the technology far outweigh the risks. In genetic engineering, cloned DNA sequences that confer resistance to the pathogen are used to transform the selected cultivar using one of several available plant transformation methods (Hille *et al*., 1989). Although this approach also requires initial investment in recombinant DNA technology, it is now the approach of choice, especially in cases where the gene is not available in the gene pool, and so it complements the classical breeding programs of the past. Genetic engineering for disease resistance involves the modification of the genetic constitution of an organism to confer the characteristic of resistance to the disease. This may involve the transfer of a fragment of DNA from the donor organism to the recipient organism, or the deletion or inactivation of a gene for susceptibility to the disease.

2.5.2 Control of viral diseases

To-date, there are as many as 675 plant viruses known and yet annual crop losses due to viruses are valued at US$60 billion (Fields 1996). There are various ways of controlling viral diseases such as:

- The use of disease-free planting material. Virus-free stocks are obtained by virus elimination through heat therapy and/or meristem tissue culture. This approach is effective for seed-borne viruses, but is ineffective for viral diseases transmitted by vectors.

- Adopting cultural practices that minimize epidemics, for example by crop rotation, quarantine, rouging diseased plants and using clean implements. Pesticides may also be used to control viral vectors, but the virus may be transmitted to the plant before the vector is killed.
- Classical cross protection, in which a mild strain of the virus is used to infect the crop, and protects the crop from super-infection by a more severe strain of the virus.
- Use of disease resistant planting material. Natural resistance against viruses may be bred into susceptible lines through classical breeding methods or transferred by genetic engineering.
- Engineered cross protection. This involves integration of pathogen-derived or virus-targeted sequences into DNA of potential host plants, and conveys resistance to the virus from which the sequences are derived.

Of all the methods of controlling viral diseases listed above, engineered cross protection seems to have a lot of potential that is only now beginning to be exploited. Before genetic engineering techniques were more widely accepted and applied, natural disease resistance genes bred into target cultivars by classical breeding methods constituted the major focus for introducing disease resistance into plants.

There are 139 monogenic and 40 polygenic virus resistance traits that have been described (Khetapal *et al*., 1998; Hull 2001), but very few have been cloned, and in most cases the mechanism of resistance has not been elucidated (Ellis *et al*., 2000; Dinesh-Kumar *et al*., 2000).

Virus-resistant crops that have been obtained by classical breeding include sugarcane resistant to sugarcane mosaic potyvirus (SCMV) and gerkins (cucumber) resistant to cucumber mosaic virus

(CMV). The *N*-gene of *Nicotiana glutinosa* that is responsible for the necrotic local lesion reaction of TMV, has also been bred into some *N. tabacum* lines, resulting in the hypersensitive reaction and no systemic infection. Classical breeding has also been used to convey polygenic traits.

2.5.3 Non-viral genes

One approach to protect plants against a viral infection is by the expression of a single chain variable fragment (scFv) antibody directed against that particular virus (Tavladoraki *et al*., 1993; Voss *et al*., 1995). This has been demonstrated for the icosahedral artichoke mottle crinkled tombusvirus (AMCV) and the rod-shaped tobacco mosaic tobamovirus. However, the resistance obtained this way is not broad-spectrum resistance.

An approach that can yield broad-spectrum resistance to viral diseases is to target the inhibition of production of a product that is essential for the establishment of infection in the cell. An example is S-adenosylhomocysteine hydrolase (SAHH), an enzyme involved in the trans-methylation reactions that use S- adenosyl methionine as a methyl donor (Masuta *et al*., 1995). Lowering expression of the enzyme suppresses the 5'-capping of mRNA that is required for efficient translation. Overexpression of cytokinin in crops results in stunting. This phenotype may be due to induction of acquired resistance (Masuta *et al*., 1995).

Expression of the pokeweed (*Phytolacca americana*) antiviral protein (PAP), a ribosome-inhibiting protein (RIP), in plants protects the plants against infection by viruses (Ready *et al*., 1986; Lodge *et al*., 1993). In this case, expression of this single gene in the plant results in protection against a wide range of plant viruses.

2.6. Pathogen-derived Resistance

2.6.1 Definition

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 & 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.

2.6.2 Classical cross protection

Pathogen derived resistance is an extension of the phenomenon of "cross protection" in which inoculation of a host plant with a milder strain of a pathogen can protect the plant from superinfection by more severe strains of the same or a very closely related pathogen (Wilson 1993). An example of cross protection is in tobacco where infecting tobacco plants with the U1 strain of tobacco mosaic tobamovirus (TMV) protects the plants against future infections with a more virulent strain of TMV.

In practice, the protected plants usually become superinfected, and so the definition given above is not practical. For practical purposes, cross protection is still defined by an earlier definition as "the use of a virus to protect against the economic damage by severe strains of the same virus" (Gonsalves & Garnsey, 1989). Classical cross protection, according to this practical definition, has been evaluated in the field in some countries outside Africa for the control of citrus tristeza

closterovirus (CTV), papaya ringspot potyvirus (PRSV), zucchini yellow mosaic potyvirus (ZYMV) and cucumber mosaic cucumovirus (CMV) (ibid).

2.6.3 Engineered Protection

The genetic engineering approach to cross protection was first demonstrated by Powell-Abel and co-workers who expressed the TMV coat protein gene in transgenic plants and obtained some degree of resistance against TMV (Powell-Abel *et al*., 1986). Many viral genes and gene-products have since been shown to be effective in conveying engineered PDR. Engineered PDR can be divided into protein-based PDR (coat protein-, replicase- and movement protein-mediated resistances, using these proteins in their wild type or defective forms) and nucleic acid-based PDR (antisense, sense and satellite RNA-mediated resistances, defective interfering RNA or DNA and antiviral ribozymes).

In general, when classical cross protection is incomplete, smaller lesions than in control non-protected plants are formed, indicating reduced movement and maybe reduced replication as well. On the other hand, transgenic plants engineered to confer protection to TMV show no reduction in movement or replication. However, the local lesions for PDR against PVX indicate a reduction in virus replication and movement (Hemenway *et al*., 1988). This demonstrates the similarity between classical and engineered protection.

The phenotype of PDR varies from delay in symptom development, through partial inhibition of virus replication, to complete immunity to challenge virus or inoculated viral RNA (Wilson, 1993; Baulcombe, 1996). Even a simple delay in symptom development could be useful if it allows plant biomass, seed or fruit development to outpace disease development.

2.6.3.1 Coat Protein-Mediated Resistance

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 (Table 2). The resistance requires that the CP transgene be transcribed and translated. Hemenway and co-workers (1998) have demonstrated direct correlation between CP expression level and the level of resistance obtained. The case of CP-MR to TMV is considered in detail below since 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 1988 and Powell *et al*., 1990).

2.6.3.1.2 CP-MR to TMV

Research showed that TMV CP transgenic plants are either immune or display delayed systemic symptom development following inoculation with TMV (Powell-Abel *et al*., 1986). The plants that resist infection were shown to lack TMV accumulation in uninoculated leaves (Nelson *et al*., 1987). When challenged with a local lesion-producing strain of TMV, transgenic *Nicotiana tabacum* cv Xanthi plants had fewer local lesions than control non-transgenic plants. The local lesions that developed, however, were just as big as on control leaves. This suggested that CP transgenic plants resist initial infection rather than subsequent replication.

Research to investigate the mechanism of resistance suggests a protein-mediated mechanism rather than an RNA-mediated mechanism (Register *et al*., 1988; Powell *et al*., 1990). A series of constructs generating mRNA sequences that would produce no CP, or mRNA that locked the

replicase recognition site but would produce CP, were utilized. It was also shown that the 3' tRNA-like sequence was not necessary to generate resistance (Powell *et al.*, 1986). Protoplasts made from CP-expressing transgenic plants were protected against infection (Register & Beachy, 1988). Tobacco protoplasts that took up CP were transiently protected from TMV infection, and CP outside the cell was probably not involved (Register & Beachy, 1989). Pathogenesis-related proteins do not appear to be involved. Protection decreases with increasing inoculum levels (Nejidat & Beachy 1990; Bendahmane *et al*., 1997b). The plants are not protected against inoculation with viral RNA (Nelson *et al*., 1987). A direct correlation was observed between the sequence similarity of transgene CP and challenging virus, with the level of protection obtained (Najidat & Beachy, 1990). TMV CP transgenic plants were better protected against tomato mosaic tobamovirus (ToMV, 82% identity) and tobacco mild green mosaic tobamovirus (TMGMV, 72% identity) than against ribgrass mosaic tobamovirus (RMV, 45% identity), and there was little or no protection against viruses from other families.

2.6.3.1.3 CP-MR to other viruses

Some of the CP-transgenic plants that exhibit CP-MR are listed in Table 1 below.

Table 3: List of transgenic plants for which coat protein-mediated resistance has been evaluated against specified viruses.

Source of CP	Transgenic Plant (TP)	Virus resistance (Spectrum)	Reference
AMV	Tobacco	AMV	Loesch-Fries *et al*., 1987
	Tomato	AMV	Turner *et al*., 1987
	Tobacco	AMV	Beachy *et al*., 1990
	Tobacco	AMV	Beachy *et al*., 1990
	Alfalfa	AMV	Beachy *et al*., 1990
CMV	Tobacco	CMV	Namba *et al*., 1991; Quemada *et al*., 1991
TSV	Tobacco	TSV	Beachy *et al*., 1990
PVX	Tobacco	PVX	Hemenway *et al*., 1988
	Potato	PVX	Beachy *et al*., 1990
	Potato	PVX	Beachy *et al*., 1990
PVY	Potato	PVY	Perlak *et al*., 1994
SMV	Tobacco	TEV, PVY	Beachy *et al*., 1990
TRV	Tobacco	TRV, PEBV	Beachy *et al*., 1990
TMV	Tobacco	TMV	Nelson *et al*., 1988
	Tobacco	ORSV, PMMV, TMGMV	Beachy *et al*., 1990
	Tomato	TMV, ToMV	Beachy *et al*., 1990
ToMV	Tomato	ToMV	Sanders *et al*., 1992
PLRV			Kawchuk *et al*., 1991
PVY	Potato	PVX, PVY, PLRV	Tacke *et al*., 1996
PVS	Potato	PVS and	MacKenzie *et al.,* 1991
PVS/PVM	Potato	PVM, weaker resistance	MacKenzie *et al*., 1991
AMV	Alfalfa	AMV	Hill *et al*., 1991
RSV	Rice	RSV	Hayakawa *et al*., 1992
ZYMV	Cantaloupe	ZYMV	Fang & Grumet, 1993
MDV	Corn	MDV, MCMV,	Murray *et al*., 1993
TSWV	Tomato	TSWV	Kim *et al*., 1994, Ulzen *et al*., 1995 Kunik *et al*.,
TYLCV		TYLCV	1994
CMV	Petunia	CMV	Kim *et al*., 1995
PPV	Plum	PPV	Scorza & Ravelonandro, 1996
PPV	Apricot plants	PPV	Machado *et al*., 1993
CTV	Citrus plants	CTV	Gutierrez-E. *et al*., 1992; Moore *et al*., 1993
CaMV	Rapeseed	CaMV	Herve *et al*., 1993
CaMV	Cauliflower	CaMV	Passelegue & Kerlan, 1996
GCMV	Grape plants	GCMV	Le Gall *et al*., 1994
GFLV	Grape plants	GFLV	Krastanova *et al*., 1995, Mauro *et al*., 1995
ToRSV	Grape plants	ToRSV	Scorza *et al*., 1996
TSWV	Chrysanthemum	TSWV	Urban *et al*., 1994; Yepes *et al*., 1995
TMV	Chinese cabbage	TMV	Jun *et al*., 1995
BYDV	Wheat	BYDV	Hansen *et al*., 1995
WSMV	Wheat	WSMV	Hansen *et al*., 1995
TSWV	Peanut	TSWV	Brar *et al*., 1994
BPMV	Soybean and bean	BPMV	Di *et al*., 1996
BGMV		BGMV	Aragoa *et al*., 1996
LMV	Lettuce	LMV	Zerbini *et al*., 1995
TSMV	Lettuce	TSMV	Pang *et al*., 1996
CMV	Sweet pepper	CMV	Zhu *et al*., 1996

The CP genes can be pyramided by themselves or to other genes to give multiple resistances. This has been done for TMV/SMV CP and TEV NIa proteinase (Marcos & Beachy 1997), *N. benthamiana* with *N* gene of TSWV and CP of TuMV (Jan *et al*., 2000a), squash expressing the CPs of CMV, WMV-2 and ZYMV (Fuchs *et al*., 1998).

2.6.3.1.4 Mechanism of CP-MR protection

Experiments with TMV CP-transgenic plants have indicated some possible mechanisms of CP-MR. Once local infection takes place in TMV CP-transgenic plants, there is no inhibition of cell-to-cell movement of the virus (Register *et al*., 1988). But if a leaf-bearing stem segment from a TMV CP transgenic plant is grafted between lower and upper sections of a non-transgenic plant, systemic movement of TMV into leaves above the graft is inhibited.

Tobacco mosaic virus particles treated at pH 8.0 or viral RNA overcomes CP-MR. The alkaline treatment greatly enhances translatability (Register 1988; Register & Beachy 1988). Using TMV-like pseudoparticles encoding β-glucuronidase (GUS), Osbourn and co-workers (1989b) showed that GUS gene expression in protoplasts from CP-transgenic plants was 100 times less efficient than control protoplasts, and most pseudo-particles (97%) remained coated.

Involvement of an early uncoating event was demonstrated by Matthews (1991). The TMV mutant DT-IG, unable to produce CP and therefore unable to move systemically, was used to inoculate CP transgenic plants. The particles were found to have moved systemically, even though the re-isolated particles were not infective on non-transgenic plants.

Osbourn and co-workers (1989a) produced double-transformed tobacco plants expressing TMV CP gene and the CAT reporter gene whose transcript contained a copy of the TMV origin of

assembly sequence. No rods could be detected by electron microscopy in cell extracts of these plants, and there was no significant CAT activity. It is therefore unlikely that re-encapsidation of uncoated challenge RNA by endogenous CP is involved in CP-MR.

Wu and co-workers (1990) compared TMV co-translational disassembly of CP(+) and CP(-) protoplasts. TMV recruited poly-ribosomes within 5 – 10 minutes and input virus was undetectable within 60 minutes in CP(-) protoplasts, whereas in CP(+) protoplasts, input virus was not recruited to poly-ribosomes and was intact at 60 minutes. This also shows CP-MR to be an early event after entry, and that the CP from the transgene may prevent disassembly.

A general hypothesis is that virions may also fail to disassemble because the presence of large quantities of CP in the cytoplasm in a way act to indicate that the infection cycle is already at the virion packaging stage, and so it does not allow disassembly of incoming virions. A specific hypothesis as to how disassembly is blocked is that of inhibition of viral uncoating (Register and Nelson 1992; Bendahmane *et al*., 1997), with the transgenically expressed CP driving the reaction towards assembly. However, inhibition of uncoating alone seems to be insufficient to account for CP-MR (Osbourn *et al*., 1989b). Clarke and co-workers also showed that the hypothesis that a cellular site for TMV disassembly is blocked by transgenic CP was not credible, by using sunn-hemp mosaic tobamovirus (SHMV)/TMV chimeric viruses where similar resistances were observed irrespective of which sequences were on the surface of the CP (Clarke *et al*., 1995).

There appears to be exceptions to observations made to the TMV system. While TMV, AMV and TRV - CPs do not protect against RNA inocula (Loesch-Fries *et al*., 1987; van Dun *et al*., 1987; Angenent *et al*., 1990), plants expressing high levels of PVX CP were resistant to infection

with PVX RNA (Hemenway *et al*., 1988). The observations in the PVX CP-transgenic plants suggest an RNA-mediated mechanism.

Statistical analysis of primary transformants is usually not done because there is a high degree of variation due to the position effect (Nap *et al*., 1993). In *Solanum dalcamara* L. *GUS* gene transformants, the *GUS* gene was silenced in 9.2% of the transformants (Curtis *et al*., 2000). It might also be interesting to look for such events in lines originally intended for CP-MR.

2.6.3.2 Replicase-mediated resistance

The viral replicase is the RNA-dependent RNA polymerase. Expression of the viral replicase gene in a transgenic plant has been shown to confer near immunity to the virus from which the replicase gene is derived. This resistance is highly specific and is usually restricted to the virus from which the replicase gene is derived. This was first demonstrated by Golemboski *et al.* (1990) who transformed *N. tabacum* with the 54 kDa read-through region of the replicase gene of the U1 strain of TMV. The plants were resistant to infection with TMV U1 strain or its RNA at high concentrations, and to a mutant of TMV U1 strain, but not to two other tobamoviruses or CMV. Resistance is due to an early event, but nucleic acid mechanisms are probably involved (Carr *et al*., 1994; Marano & Baulcombe 1998).

Replicase-mediated resistance is characterized by a high degree of resistance to both viral and RNA inocula, and high specificity. In most cases, there is a substantial inhibition of virus replication in the initially inoculated cells and some limited cell-to-cell movement, but infection does not spread from the inoculated leaf, and no systemic disease develops. In fact, this could be referred to as immunity.

Effects of the transgene may include one or more of the following: suppression of virus replication; inhibition of long distance transport resulting from inhibition of entry into the vascular system; or lack of cell-to-cell movement as a result of shut-down of plasmodesmata. Monitoring of RNA levels seems to suggest an RNA-mediated mechanism, as is the case with PVX replicase in tobacco (Baulcombe, 1994b). Evidence for a protein-mediated mechanism is provided by the observation made by Brederode and co-workers (1995) that transgenic alfalfa plants expressing the highest levels of AMV replicase have the highest levels of resistance. Furthermore, *in vitro* studies showed that the TMV 54-kDa resistance supports a protein-mediated mechanism since there is a requirement for a functional protein (Carr *et al*., 1992). It is therefore possible that different mechanisms may be at work in different systems.

Replicases of TMV, PEBMV, PMMV, PVX, CyRSV, CMV, AMV, PVY, CPMV and PLRV have been used either as wild type or mutated forms to convey resistance (Zaitlin, 1994). The AMV P1 or P2 replicase genes failed to give protection in transgenic plants, neither did the N-truncated P2 gene (to resemble TMV 54 kDa) or P2 gene with the gly-asp-asp (GDD) motif changed to val-asp-asp (VDD) (Brederode *et al*., 1995). However, mutation to GGD, GVD or DDD gave higher levels of protection against AMV. AMV, CMV and TMV systems show relatively high steady-state levels of transgene mRNA. Truncated protein was detected, but effectiveness increased with increased translatability only for CMV (Carr *et al*., 1994; Wintermantel & Zaitlin 2000). Wintermantel & Zaitlin (2000) working with the 2a replicase gene of the Fny strain of CMV showed that translatability of the transgene, and possibly expression of the transgene protein itself, facilitates replicase-mediated resistance in tobacco. Mutations in the TMV and AMV replicase protein sequences interfered with protection (Carr & Zaitlin 1992; Brederode *et al*., 1995). These results suggest a protein-mediated protection mechanism. Both full length and truncated forms of the replicase gene have thus been used, but

the full length and functional replicase genes do not always lead to resistance (Donson *et al*., 1993; Golemboski *et al*., 1990).

2.6.3.4 Movement protein-mediated resistance

Movement protein-mediated resistance (MP-MR) was first demonstrated by Malyshenko and co-workers (1993) who expressed the temperature sensitive movement protein (MP) of TMV in tobacco and showed that the TMV MP transgenic plants were relatively resistant to TMV. Expression of a dysfunctional MP of a virus in transgenic plants has been shown to confer resistance to that virus and other related and unrelated viruses (ibid). Virus movement is reduced within the infected plant.

Transgenic plants expressing the TMV MP with a 3 amino acid deletion also showed reduced local and systemic spread (Lapidot *et al*., 1993). Other workers expressed a defective p13 protein of *White clover mosaic potexvirus* (WCMV) strain O (Beck *et al*., 1994 and Seppanen *et al*., 1997). The p13 protein is one of the triple block proteins implicated in local spread of potex viruses. They demonstrated resistance to WCMV strains O, M and J, and also *potato S carlavirus* (PVS), but no resistance to TMV.

Cooper and co-workers (1995) showed that TMV MP transgenic tobacco plants were resistant to several tobamoviruses, and also *Tobacco rattle tobravirus* (TRV), *Tobacco ringspot nepovirus* (TRSV), *Alfalfa mosaic alfamovirus* (AMV), *Peanut chlorotic streak caulimovirus* (PCSV), CMV, PVY and TRV. In non-tobamoviruses, the defective MP (dMP) allowed infection and local spread, but no systemic infection was established. However, tobacco plants expressing the functional wild type TMV movement protein accelerated symptom development and symptom severity and increased the accumulation of these viruses and TMV. It would have been

interesting to investigate how this response varies with varying levels of MP. Hou and co-workers (2000) reported that the MP of bean dwarf virus (BDMV) has deleterious effects on plant development.

The mechanism by which MP-MR is thought to act is by interference of function at the level of multi-protein interactions. This may be achieved at the level of targeting of the protein to the cell wall or plasmodesmata, or by interference with association of the MP with nucleic acids. Some other mechanism, however, may also be effective. The dysfunctional MP prevents the accumulation of the MP of challenge virus in such a way that the latter fails to complete its function. This is consistent with the observation that the transgenic plants expressing the functional MP accelerate local and systemic spread of heterologous viruses against which dMP mediated resistance effective. Thus, even when a wild type MP confers resistance to a particular virus, it is unwise to use this as it might result in increased susceptibility to other viruses. It is still to be demonstrated whether highly resistant or immune plants can be obtained through this approach.

2.6.3.5 Antisense RNA-mediated resistance

When a gene is cloned in a reverse orientation, and integrated into the plant genome, its complementary strand will be transcribed into mRNA. If this occurs, and results in resistance, this is what is known as antisense-mediated resistance (AR-MR), also called messenger RNA interfering complementary RNA (micRNA), (Melster and Tuschl, 2004)

2.6.3.5.1 Antisense RNA against plant RNA viruses

Antisense RNA can act *in trans* against multiple gene copies, and therefore appears to be an ideal tool for suppressing viral infections. However, more than 98% of plant viruses have RNA

genomes, and probably replicate in the cytoplasm. If the mechanism of antisense-mediated resistance rely on interactions in the nucleus, this limits the number of stages at which viral replication can be blocked. If these interactions were to occur in the cytoplasm, the large volume of the cytoplasm may result in an increase in the time required for duplex interactions. The antisense RNA molecules may therefore be degraded by RNases before encountering a target viral RNA. Antisense RNA approaches have therefore not been very successful against plant viruses, especially antisense CP RNA. Protection was observed only when low levels of challenge inoculum was applied. Coat proteins do not seem to be the best targets for antisense-mediated resistance since they are expressed at high levels, and coat proteins are not always essential for virus replication and symptom development.

Antisense RNA-mediated resistance has not been very successful against various regions of CMV (Cuozzo *et al*., 1988) and TRV. PLRV gave higher resistance levels, similar to sense, (and <10% of control). Field resistance to TSWV in transgenic peanut expressing antisense nucleocapsid gene sequence has also been reported (Magbanua *et al*., 2000).

2.6.3.5.2 Antisense RNA against plant DNA viruses

Caulimoviruses and geminiviruses are two important plant DNA virus groups. Caulimoviruses have double-stranded, circular DNA genomes that replicate through an RNA intermediate and have a narrow host range.

Geminiviruses, exemplified by tomato golden mosaic virus (TGMV) have twinned, quasi-icosahedral particles containing a small, circular ssDNA molecule of about 2.6 kb. They have bipartite genomes and they infect both monocots and dicots. Geminiviruses accumulate in the nuclei and are a good target for study. The antisense strategy has been effective against gemini

viruses, for example, antisense CP of TGMV (Frischmuths & Stanley, 1993), replicases of TGMV and TYLCV; (Day *et al*., 1991), and CP of tomato mottle bigeminivirus (ToMoV) (Sinisterra *et al*., 1999).

2.6.3.5.3 Other aspects of antisense RNA-mediated resistance

In vitro experiments showed that oligonucleotides complementary to genomic PVX RNA caused translational arrest in Krebs-2 cell-free system, probably due to RNase H activity (Miroshnichenko *et al*. 1988). Crum and co-workers (1988) used antisense sequences to the 5' end of TMV RNA to inhibit TMV translation in a rabbit reticulocyte lysate. Inhibition was probably due to direct interference with ribosome attachment (ibid).

The antisense approach has been successful against BYMV (Hammond & Kano, 1995) and TRSV (Yepes *et al*., 1996). The success of the antisense approach to TRSV was probably attributed to a sense RNA-mediated mechanism (Yepes *et al*., 1996). The antisense approach has also given protection against some viroids (Tabler *et al*., 1998).

Antisense RNA molecule is long and complexes readily with proteins. Low RNA levels are effective while high levels are not effective in conveying resistance (Smith *et al*., 1995). It is therefore unlikely that the mechanism of resistance is by hybridisation of sense to antisense RNA alone.

There are anti-sense RNAs that are naturally transcribed in plants (Terryn & Rouze, 2000). These may originate as overlapping transcripts, encoding a peptide, or may be involved in regulation of the sense transcript. The silencers and enhancers in the introns (non-viral systems) may therefore actually control anti-sense RNA production. The anti-sense RNA forms double-

stranded RNA that affects RNA stability, transcription or translation, and may generate a signal for gene silencing and defence against viruses (ibid).

There are several proposed mechanisms of action by which antisense-mediated resistance is achieved (Terryn and Rouze, 2000). The anti-sense mRNA may hybridise to the genomic DNA template of a DNA virus to result in a DNA/RNA double stranded molecule, and block sites of transcription. The RNA strand of DNA/RNA hybrid may also be degraded by RNase H. Anti-sense RNA may also bind to the sense pre-mRNA to result in an antisense RNA:mRNA duplex. This may result in blocking of intron splicing. The antisense RNA:mRNA duplex is in fact double stranded RNA and may fail to be transported from the nucleus into the cytoplasm. The dsRNA may block initiation of translation, and also trigger a mechanism for rapid RNA degradation either in the nucleus or the cytoplasm. Effectively, actual mechanisms may be similar to those of sense RNA-mediated protection discussed later.

2.6.3.6 Sense RNA-mediated resistance

It has also been observed that in some cases where the CP gene (or part thereof) is introduced into the plant in the sense orientation, resistance is obtained without the transcribed CP mRNA being translated into a protein. Observations that no construct without a promoter could confer resistance (Lomonossorf, 1995), led to the deduction that resistance is either due to transcribed RNA or to the protein translated from the RNA.

Unexpected results in CP-MR experiments included the lack of correlation between resistance and the expression of PVY or PLRV CPs (Kawchuk *et al*., 1990; Lawson *et al*., 1990). In TEV, Lindbo & Dougherty (1992) found that the untranslatable CP gene of TEV gave higher levels of protection than the full length or truncated translatable constructs. Similar observations were

made for TSWV (de Haan *et al*., 1992) and PVY (van der Vlugt *et al*., 1992). These results indicate that there might be a mechanism of protection at the level of nucleic acids.

Not all the plant lines expressing a non-coding RNA show protection. According to Smith and co-workers (1994) there are several features that characterize RNA-mediated protection. There is no direct correlation between RNA expression level and the level of protection (Pang *et al*., 1993). Usually no transgene-encoded protein is detectable, and the steady-state level of its transcript in challenged plants is low. The range of protection is narrow, being effective only against viruses with sequences similar to transgene. Protection is not overcome by challenging with viral RNA. Protection is not dose-dependent, and operates at high levels of inoculum. Copies of the transgene may be comprised of multiple copies of the transgene, particularly with direct repeats of coding region (Sijen *et al*., 1996) or may be truncated or in an antisense orientation (Waterhouse *et al*., 1998; Kohli *et al*., 1996). The transgene sequences and sometimes their promoter(s) may be methylated (Jones *et al*., 1999; Kohli *et al*., 1996; Sonoda *et al*., 1999). The transgenic plants can be classified into three groups (Smith *et al.,* 1995). The first group is comprised of susceptible plants. These plants have low to moderate levels of transgene transcription and steady state RNA. The second group is comprised of plants that initially become infected and then recover. These plants have moderate to high levels of transgene transcription and steady-state RNA in uninfected plants, but low-level steady-state RNA in recovered tissues. The third and final group is that of highly resistant plants that have high levels of transgene expression but low steady-state RNA levels.

Sijen and co-workers (1995) showed that the CPMV MP protects transgenic cowpea plants by an RNA-based mechanism, and the mechanism also operates against PVX RNA containing CPMV MP. Frequency of resistant lines was increased 20 – 60 % by using a direct repeat of the MP

gene. Maiti and co-workers investigated pyramiding TVMV CP, NIa and NIb genes in transgenic plants (Maiti *et al.*, 1999). Plants with the combination of all the three genes were less resistant to TVMV than those expressing the CP gene alone, and were not resistant to TEV.

Immunity to barley yellow dwarf luteovirus (BYDV-PAV) was conferred to barley plants transformed with the hairpin RNA containing the polymerase gene of BYDV (Wang *et al*., 2001a). Sense RNA-mediated resistance is actually a possible mechanism for cross protection. Tomato ringspot nepovirus (ToRSV) has a bipartite genome of two single-stranded positive sense RNA molecules separately encapsidated. ToRSV has a broad host range, and when used in resistance studies, sense and antisense constructs gave similar levels of resistance. The mechanism of resistance is likely to be a post-transcriptional RNA degradation process (Goodwin *et al*., 1996). A line expressing an untranslatable mRNA of TEV CP open reading frame, with three distinct transgene integration events that segregated as two linkage groups, was studied. A genetic series of plants that produced 0, 1, 2 or all 3 transgene inserts in the homozygous or heterozygous conditions were examined. Genetic and biochemical data suggest that RNA-mediated virus resistance is a multigenic trait in line 2RC-6.13, a line transgenic for the untranslatable TEV CP mRNA. Three or more transgenes were necessary to establish the highly resistant state. One or two copies resulted in an inducible form of resistance, which is equivalent to the recovery phenotype. Transcription rates and steady-state RNA levels of the transgene-derived transcripts present in different members of the genetic series supported the notion that a post-transcriptional RNA degradation process is the underlying mechanism for transgene transcript reduction and ultimately, virus resistance. This degradation process appeared to initiate via cleavage of specific sites within the target RNA sequence, as determined by RNA gel blot and primer extension analyses of transgene-derived mRNA from various transgenic plant lines.

Lines accumulating higher levels of transgene RNA may be susceptible while those with lower levels are resistant. Hybridisation with minus strand intermediates synthesized during PVY replication is therefore an unlikely mechanism (Smith *et al*., 1995). Also, the transgene RNA is unlikely to titrate out any essential host or viral factors essential for viral replication.

RNA-mediated resistance is sometimes called cell-mediated response because viral RNA activates a host response that degrades viral RNA (Smith *et al*., 1995). This phenomenon is characterized by high transcription rates and low RNA steady-state levels. This is a result of the pre-activated sequence-specific degradation pathway for aberrant or over expressed RNAs. Since it is pre-activated, low levels of transgene mRNA results in high resistance. The mechanism by which this is achieved is therefore a homology-dependent gene silencing (HDGS) mechanism

Homology-dependent gene silencing, HDGS, has been observed in higher plants, fungi, *Drosophila* and mammalian cells (Smith *et al*., 1995), and appears to be a general protection mechanism in multi-cellular organisms. This HDGS is divided into transcriptional gene silencing (TGS) and post-transcriptional gene silencing (PTGS). Transcriptional gene silencing is often associated with DNA methylation, and generally requires promoter homology. Post-transcriptional gene silencing is characterized by active transcription, shown by run-on transcription assays, but low or no accumulation of mRNA.

Post-transcriptional gene silencing is a nucleotide sequence-specific defence mechanism that can target both cellular and viral mRNAs (Hamilton & Baulcombe, 2000). Short antisense RNA molecules, about 25 nucleotides long, may be involved, and accumulate in response to either transgene sense transcription or RNA virus replication. The 25-nucleotide antisense RNA is therefore likely to be synthesized from an RNA template and may represent the specificity

determinant of PTGS. Post-transcriptional gene silencing is manifested as the reduction in steady-state levels of specific RNAs after the introduction of homologous sequences in the plant genome. This reduction is a result of increased turnover of target RNA species with the transcription level of the corresponding genes remaining unaffected. The silencing signal directs sequence-specific RNA degradation, and is spread systemically (ibid).

In PTGS abnormal RNAs are also observed. These are truncated RNAs resulting from premature termination, degradation intermediates, or RNAs containing antisense regions. Abnormalities potentially act as degradation signals, and trigger degradation of all transcripts from the transgene. However, no direct evidence for this has been obtained (Kanno *et al*., 2000).

Current efforts in molecular biology are aimed at further elucidating the mechanisms by which RNA interference is effected. It is hoped that there is a single basic mechanism for both plants and animals. If a common mechanism for silencing exists, it cannot be at the level of methylation since methylation is independent of RNA interference in some organisms, such as *Neurospora* (Freitag *et al*., 2004). Recently, Herr and co-workers (2005) cloned and characterized the large subunit of RNA polymerase IV, unique to plants, that is involved in a short interfering RNA pathway involving RNA-dependent RNA polymerase II and Dicer-like 3 (ibid). Long terminal repeats (LTRs) scattered over plant genomes are thought to be involved in regulation of gene expression, even though they are remnants of past transposon activity (Comfort, 2003). Hairpin RNAs and retrotransposon LTRs have been shown to result in RNA interference and ultimately euchromatin-to-heterochromatin change, effectively silencing expression of genes with common sequences to the hairpin RNA or LTR, and those surrounding them (Schramke & Allshire, 2003). However, heterochromatin formation is not always dependent on RNAi pathways (Jia *et al*., 2004).

Oilseed rape (*Brassica napus*) has been shown to respond to cauliflower mosaic virus systemic infection by both TGS and PTGS mechanisms to enhance and subsequently suppress viral gene expression (Al-Kaff *et al*., 2000).

The phenomenon of PTGS is well reported in dicots, but has only recently been reported in monocots (Kanno *et al*., 2000). Transcription run-on experiments were used to demonstrate that the PTGS in rice protoplasts was by a homology-dependent suppression.

2.6.3.7 Protein and RNA-mediated protection

In a number of cases, the same construct is thought to confer resistance by both protein and RNA-mediated mechanisms. Using the TSWV *N* gene, although protection against heterologous tospoviruses was observed in plants with the highest transgene protein levels, the most effective homologous protection in plants with lowest steady-state levels of RNA and little protein (Pang *et al*., 1993). BYDV-PAV CP (McGarth *et al*., 1997) and TMV replicase sequences (Goregaoker *et al*., 2000) are also thought to protect plants by a protein- as well as sense RNA-mediated mechanisms. The PMTV CP in *N. bethamiana* resulted in strong resistance to virus irrespective of the amount of RNA or protein detected (Barker *et al*., 1998). The mutated PLRV orf 4 (MP) in potato plants results in protection against PLRV (RNA-mediated) and PVX and PVY (protein-mediated). Palukaitis & Zaitlin (1997) advise that all transformants be tested, not only those with the best protection.

2.6.3.8 Satellite virus RNA-mediated resistance

Satellite RNAs (sat-RNAs) are parasitic RNAs that need a helper virus to replicate in host plants but have no sequence homology with the helper virus (Simon, 1988). Sat-RNAs can either attenuate or enhance the symptoms of the helper virus. Baulcombe and co-workers (1986)

expressed the CMV sat-RNA in plants. Large amounts of transgene sat-RNA were produced in the plants upon infection with a satellite-free CMV inoculum, and the sat-RNA became transmissible with the virus. The sat-RNA decreased CMV replication and largely suppressed symptoms. Tomato aspermy cucumovirus (TAV) also induced CMV sat-RNA synthesis accompanied by symptom suppression but with little decrease in TAV replication (Harrison *et al.*, 1987). The CMV sat-RNA also resulted in delayed disease development against TMV (Kim *et al.*, 1995). Gerlach and co-workers (1987) showed that the TobRV Sat-RNA in transgenic plants conveys resistance to TobRV. Protection was maintained for life.

Harrison and co-workers (1987) showed that tobacco plants transgenic for the CMV sat-RNA and challenged with satellite-free CMV performed almost as well as control unchallenged plants. Similar results were obtained when CMV was introduced via aphids (Jacquemond *et al*., 1988). Taliansky and co-workers (1998) made transgenic *N. benthamiana* expressing a mild satellite GRV variant. At least two mechanisms were shown to be at work when the transgenic plants were challenged with GRV carrying severe sat GRV.

The advantages of transgenic sat-RNA-MR are that, first, protection is better than CP-MR in that it does not depend on inoculum concentration. Second, only those plants that become naturally infected in the field will become stunted, unlike in cross protection. Protection is stronger than cross protection, and there is no need for inoculation every season. The disadvantages are that the sat-RNA may cause virulent disease in other crop species, and could easily mutate to a disease-enhancing form (Sleat and Palukaitis, 1992). Also, the protected plants serve as a reservoir of virus, and the approach is only limited to those viruses for which symptom-reducing sat-RNAs are known. In practice, the use of sat-RNA may not be very practical because only a

few base changes are required to change a symptom-reducing sat-RNA to a symptom-enhancing sat-RNA (Sleat and Palukaitis, 1992).

2.6.3.9 Defective interfering nucleic acid-mediated resistance

Defective interfering (DI) nucleic acids are naturally occurring mutants of viral genomes, sharing sequence homology, but are incapable of autonomous replication even though they contain sequences that enable them to be replicated with, and sometimes at the expense of, the parent helper virus (Hull, 2001). This may result in amelioration of the symptoms of the parent virus.

Tobacco plants expressing the *Tomato bushy stunt tombusvirus* (TBSV) DI RNA were protected against TBSV and related tombusviruses *Cucumber necrosis tombusvirus* (CNV) and *Carnation Italian ringspot tombusvirus* (CIRV), but were susceptible to distantly related *Cymbidium ringspot tombusvirus* (CymRSV) and unrelated BDMV, PVX and TMV (Rubio *et al*., 1999). Transgenic *N. benthamiana* plants expressing the DI DNA of the Logan strain of *Beet curly top hybrigeminivirus* (BCTV) were protected from this particular strain, with reduced virus accumulation, but not against other strains of the virus. DI RNA was shown to act as competitive inhibitors of the replication of TYMV (Valle *et al.*, 1989). The transgenic plants expressed the 3'-terminal region of TYMV genome, which contains the replicase recognition site. Artificial DI RNAs were also shown to interfere with corresponding BMV or CymRSV replication in *N. benthamiana* (Marsh *et al.,* 1991a; Marsh *et al.,* 1991b; Rubino *et al*., 2004). The *African cassava mosaic virus* (ACMV) DI DNA was shown to inhibit viral replication when expressed in transgenic *N. benthamiana* (Frischmuth & Stanley, 1991, 1993). Protection was to closely related viruses. The DI DNA could be serially transmitted to other plants.

2.6.3.10 Antiviral Ribozymes

Ribozymes are RNA molecules that can cut and ligate RNA or DNA molecules in a catalytic fashion (Cech & Bass, 1986). If the RNA molecule that is cut is part of a viral genome, the virus molecule may be rendered non-infective. Hammerhead ribozymes are found in sat-RNAs, viroids and virusoids where they cut multimeric and circular replication products into their linear monomers (Forster & Symons, 1987).

Since ribozymes cleave at specific sites on complementary RNA molecules, ribozymes can be considered together with antisense RNA mechanisms. Antiviral ribozymes have not improved AR-MR against TMV (de Feyter *et al*., 1996), but improved antisense-mediated resistance against PPV (hammerhead, Liu *et al*., 2000).

2.7 Mechanisms of pathogen derived resistance

The actual mechanisms that confer the observed resistances have not been conclusively demonstrated. The first mechanism of CP-MR to be proposed was interference with disassembly (Baulcombe, 1996). The CP produced in transgenic plants can somehow prevent the removal of the initial few dozen CP subunits. This also explains why naked RNA overcomes CP-MR, although conclusive and convincing experimental evidence to support this is still lacking.

The second mechanism to be considered is that of dominant negative genes. Dominant negative genes are genes with defects that are dominant over their wild type homologues (Baulcombe, 1996). More precisely, these are dominant negative alleles. For example, a virus-encoded replicase subunit with a defect can interact with host factors to assemble a defective replicase, which is unable to perform normal viral RNA replication. Since the defective gene is expressed in plants prior to viral infection, it essentially pre-occupies all available host factors and makes

them inaccessible to wild type, fully functional viral protein. Alternatively, defective viral proteins compete with wild type viral proteins for viral template RNA during replication or for viral RNA during cell-to-cell movement. The latter is the most likely mechanism for some of the observed defective replicase genes and movement gene effects.

Finally, template-specific RNA degradation is a possible mechanism of PDR. Template-specific RNA degradation is the likely mechanism for untranslated RNA (e.g. TEV, PVX). Resistance is achieved by specifically degrading viral RNA sequences that are homologous to the transgene RNA sequence. Specific methylation of the transgene sequence may or may not be involved (English *et al*., 1996; Goodwin *et al*., 1996).

By considering mechanisms of resistance it may be convenient to classify PDR into posttranscriptional gene silencing (PTGS) mechanisms and transcriptional gene silencing mechanisms (TGS). PTGS is manifested as the reduction in steady-state levels of specific RNAs after the introduction of homologous sequences in the plant genome. This reduction is a result of increased turnover of target RNA species with the transcription level of the corresponding genes remaining unaffected. The silencing signal directs sequence-specific RNA degradation, and is spread systemically.

Germinating seeds on medium supplemented with 30 μM 5-azacytidine reveals seeds in which original transgenes were inactivated by DNA methylation (Curtis *et al*., 2000). Alternatively, restriction endonucleases can be used: C-methylation insensitive e.g. *Nde*I; C-methylation sensitive, e.g. *Sau*3a. The resistances described so far are summarized in the Table 4 below.

Table 4: Summary of probable mechanisms and effectiveness of PDR.

Viral gene	Examples	Proposed mechanism	Range of protection	Comment
CP	TMV PVX	CP-MR RNA-MR	Wide Narrow	Not effective against RNA inoculum. Effective against high inoculum conc. and RNA.
Replicase	TMV PVX	Replicase protein-mediated resistance RNA-MR	Narrow	Very effective. Near immunity possible. Too narrow.
Movement protein	TMV, dysfunctional	Interference with normal function	~Narrow	Functional MP accelerates symptom development and severity.
Antisense RNA	CMV	Hybridization, Degradation, RNA-mediated	Narrow	Not very effective. Effective only against low levels of innoculum.
Sense RNA	TEV	PTGS, RNA degradation. RNA-mediated.	Narrow	Very effective. Truncated genes also effective.
Sat-RNA	TobRV sat RNA	Competition	Narrow	Protection for life. Better than cross protection and CP-MR.
DI RNA/ DNA	TBSV-DI RNA	Competitive inhibition of replication	Relatively wide	Effective
Antiviral ribozymes	Hammerhead/ PPV	RNA cleavage	Narrow	Like AS-MR, not very effective.

More likely than not, there is only one mechanism of action at nucleic acid level and another one at protein level. What remains now is data to indicate which particular model is correct for the various approaches. The mechanism at protein level seems to be based on protein-protein interaction, while that at nucleic acid level appears to be based on RNA interference.

2.8 Plant transformation

Transgenic plants are important in two respects. While they may be regarded as products that can be marketed on their own, transgenic plants are also important as tools used to study phenomena

of interest emanating from the mere presence, absence or expression of introduced or knocked-out genes in a living plant.

The methods for plant transformation can be divided into two main groups, *Agrobacterium*-mediated transformation methods, and direct gene transfer methods (micro projectile bombardment (biolistics), silicon carbide whisker-mediated transformation, electroporation and polyethylene glycol-mediated transformation (of protoplasts)). The agrolistic transformation method is a fusion of the two methods (Smith *et al*., 2001).

2.8.1 *Agrobacterium*-mediated transformation

The first reports of *in vitro* plant transformation utilised the ability of *Agrobacterium tumefaciens* to transfer a specific region of its Ti plasmid DNA into plant cells where they subsequently become integrated into the plant cell genome (Marton *et al*., 1979; Barton *et al*., 1983; Herrera-Estrella *et al*., 1983). This application is based on the observation that in natural diseases of dicotyledonous plants, crown gall disease caused by *Agrobacterium tumefaciens* and hairy root disease caused by *Agrobacterium rhizogenes*, the bacterium transfers part of the DNA of its Ti or Ri plasmid DNA respectively into the host plant where it becomes integrated into the host genome (Herrera-Estrella *et al*., 1983). The transferred DNA is referred to as the T-DNA and is demarcated by conserved left and right border sequences (ibid). The integrated genes are passed on to the progeny of the initially infected cell, and their expression (using the host's transcription and translation machinery) results in the cancerous growth that characterise the disease (crown gall or hairy root diseases). The tumours produce specific amino acid derivatives called opines that are utilized by the *Agrobacterium* as a carbon source (Zupan and Zmbryski, 1997). Within the T-DNA is a 35 kb virulence (*vir*) region that includes the genes *vir*A to *vir*R (Zhu *et al.* 2000), flanked by imperfect 25 bp direct repeat sequences known as the left and right borders. A

number of virulence genes (*chv*) located on the *Agrobacterium* chromosome mediate chemotaxis and attachment of the bacterium to the plant cell wall (Zupan & Zambryski, 1997).

In adapting the *Agrobacterium* system to genetic engineering, only the sequences that are essential for transfer and integration into the host genome have been retained, and DNA sequences of interest are inserted into the transferred DNA region. The first generation plasmids for *Agrobacterium*-mediated plant transformation were the disarmed Ti-plasmids. The oncogenes within the left and right borders of the naturally occurring plasmid pTiC58 were replaced with pBR322 sequences, to give pGV3850 (Zambryski *et al*., 1983), and further improved by the addition of a selectable marker (Bevan *et al*., 1983). Use of intermediate vectors enabled use of smaller plasmids with unique cloning sites for initial cloning experiments in *E. coli* (Matzke & Chilton 1981). The intermediate vector could be transferred from *E. coli* to *Agrobacterium* by conjugation, utilizing a helper plasmid, e.g. RK2013, to supply the requirements for conjugation (ibid). Homologous recombination between the intermediate plasmid and a resident disarmed Ti-plasmid of the *Agrobacterium* (e.g. pGV3850) resulted in a larger plasmid known as cointegrate disarmed Ti-plasmid.

In a different approach, the virulence genes were placed in a separate plasmid such as pAL4404 where these functions would be provided *in trans* for the transfer of DNA on another smaller plasmid with only the left and right borders, markers and other sequences of interest that need to be transferred such as pBin19 in the same *Agrobacterium* cell (Zupan & Zambryski, 1997). This system is known as the binary vector system. The vectors carry a broad host range replication origin, e.g. *ori V* of pBin 19, which allows replication in *E. coli* and *Agrobacterium*. The *A. tumefaciens* is used most extensively in plant transformation because of the belief that the DNA

transfer is discreet, with high proportion of integration events with single or low T-DNA copy number, compared to other methods of plant transformation (Zupan & Zambryski, 1997).

Gene disruption may occur at the site of insertion, resulting in loss of some essential functions (Birch, 1997). It is therefore important to obtain as many transformants as possible so as to be able to disregard all abnormal regenerants resulting from this or other phenomena. T-DNA transfer occurs sequentially but not always completely from the right border to the left border (Wang *et al*., 1984).

2.8.2 Direct gene transfer methods

In direct gene transfer methods a plasmid in which the sequences of interest are cloned is delivered across the various plant cell barriers by physical means to enter the cell where integration into the plant genome may occur. The vectors used in direct plant transformation methods usually include the gene of interest cloned between a promoter and a terminator, and the plasmid components of an origin of replication, an antibiotic resistance gene, a selectable marker for use in plants (e.g. herbicide or antibiotic resistance) or reporter gene (e.g. GUS, luciferase genes). The whole plasmid may be transferred into the plant cell and may be integrated into the plant genome as a whole or as fragments.

The barriers to be crossed by the DNA in direct DNA transfer methods are the cell wall and the cell membrane before it can cross the cytoplasm and the nuclear envelop to enter the nucleoplasm where the DNA may integrate into the plant genome. Some direct DNA transfer procedures utilize whole plasmids, supercoiled or linear, which may ultimately integrate wholesome, or at least large parts thereof, including the gene of interest (Smith *et al*., 2001).

Direct gene transfer methods were developed in an effort to transform economically important crops that remained recalcitrant to *Agrobacterium*-mediated transformation because of limitations such as genotype and host cell specificity. Some direct gene transfer methods may also circumvent difficult tissue culture methods.

2.8.2.1 Microprojectile bombardment

Sanford and co-workers (1987) were the first to report of plant transformation by microprojectile bombardment. Gold or tungsten particles coated with DNA are propelled at high speed toward the plant tissue where they may penetrate the plant cell walls to introduce the DNA into the cytoplasm, vacuoles, nucleus or other structures of intact cells. A modified bullet gun or electric discharge gun is used to propel the particles (Klein *et al*., 1987; Christou *et al*., 1988). Inside the cell, the DNA may be expressed transiently for two or three days before being degraded, or may become integrated into the nuclear or chloroplast genome, and considered stably integrated if it is passed faithfully to subsequent generations. DNA-coated particles delivered into the nucleus are 45 times more likely to be transiently expressed than those delivered to the cytosol, and 900 times more likely to be expressed than those delivered to the vacuole (Yamashita *et al*., 1991). Efficiency of transformation is influenced by the stage of the cell cycle (Iida *et al*., 1991; Kartzke *et al*., 1990). The DNA is also likely to be expressed if it is delivered to the cell close to the time the nuclear membrane disappears at mitosis (Bower & Birch, 1990; Vasil *et al*., 1991).

Direct DNA transfer methods seem to result in transformants with higher copy numbers than *Agrobacterium*-mediated transformation methods (Hadi *et al*., 1996; Christou *et al*., 1989). The multiple copies may be integrated at the same or tightly linked loci, most likely in relation to replication forks or integration hot spots resulting from initial integration events (Cooley *et al*., 1995, Kohli *et al*., 1998). Increasing the amount of DNA entering the cell in bombardment

increases the copy number (Smith *et al*., 2001). The DNA may undergo rearrangements (deletions, direct repetitions, inverted repetitions, ligation, concatamerization) prior to, or during integration (Cooley *et al*., 1995). The site of integration is thought to be random. Ninety percent of T-DNA integrations are into random sites within transcriptionally active regions (Lindsey *et al*., 1993).

2.8.2.2 Silicon carbide whisker-mediated transformation

In this relatively new method of plant transformation, silicon carbide crystals (average dimensions of 0.6 μm diameter, 10 – 80 μm long) are mixed with DNA and plant cells by vortexing, enabling the crystals to pierce the cell walls (Kaeppler *et al*., 1990, Songstad *et al*., 1995). This method has been used for the stable transformation of tobacco and maize (Kaeppler *et al*., 1992, Frame *et al*., 1994), and grasses (Dalton *et al*., 1998). The method appears to be widely adaptable, and can be used with as little as 0.1 μg DNA. It appears as if there is a lot of scope for further development of this method of plant transformation (Thompson *et al*., 1995).

2.8.2.3 Electroporation and PEG-mediated transformation of protoplasts

Plant cell walls can be removed by enzymatic degradation to produce protoplasts. Polyethylene glycol (PEG) causes permeabilization of the plasma membrane, allowing the passage of macromolecules into the cell. Pazkowski and co-workers were the first to produce transgenic plants after PEG transformation of protoplasts, and many more monocotyledonous and dicotyledonous species have now been transformed using this method (Pazkowski *et al*. 1984). In electroporation, the protoplasts are subjected to an electric pulse that renders the plasma membrane of the protoplasts permeable to macromolecules.

The transgenic plants generated using these methods seem to have characteristics similar to those of plants derived from all other direct transformation methods. However, it is important to note that carrier DNA (usually ~500 bp fragments of calf thymus DNA) is usually included in the transformation mixture to increase transformation efficiency. This may have some consequences in terms of prevalence of transgene rearrangements and integration of superfluous sequences (Smith *et al*., 2001).

The cell cycle stage of the protoplasts at the time of transformation influence the transgene integration pattern. Non-synchronized protoplasts produce predominantly non-rearranged single copy transgenes in contrast to M phase protoplasts that give multiple copies usually at separate loci (Kartzke *et al*., 1990). The S phase protoplasts give high copy numbers, usually with rearrangements.

Irradiation of protoplasts shortly before or after addition of DNA in direct transformation procedures increases both the frequency of transformation and number of integration sites (Koehler *et al*., 1989, 1990, Gharti-Chhertri *et al*., 1990). This is consistent with a mechanism of integration that is partly mediated by DNA repair mechanisms.

The main drawbacks of these methods are that protoplast cultures are not easy to establish and maintain, and regeneration of whole plants from the protoplasts is often unreliable for some important species.

2.8.3 Agrolistic transformation

In this method, the transforming plasmid is transferred to the plant cell by a direct mechanism together with a second plasmid coding for *A. tumefaciens* proteins involved in the integration

process (Zupan & Zambryski, 1997). Transient expression of the *A. tumefaciens* proteins will direct integration of the plasmid into the plant cell genome. As a result, entry of the plasmid into the cell is by a direct/physical mechanism, but integration into the genome is by a mechanism similar to *Agrobacterium*-mediated transformation.

2.9 Cowpea transformation

The variety of plant transformation procedures described above arose as a result of the fact that many plant species initially proved recalcitrant to *Agrobacterium*-mediated transformation. For cowpea, there is still no reliable, efficient, reproducible transformation procedure. Experiments to transform cowpea have been going on in various laboratories around the world, for example, IITA Nigeria since 1989 when a research unit dedicated to cowpea transformation was established, and more recently, T.J. Higgins at CSIRO Plant Industry, Canberra, Australia, R. Allison at Michigan State University, Ray Bressan at Purdue University, and George Bruening at California Davis (NGICA Newsletter Volume 1, No. 3, August 18, 2004; http://www.isp.msu.edu/ crsp/WorkplansforFY05/ wa5-a1.pdf).

Even though exogenous DNA could be introduced into cowpea cells, the cells could not be manipulated to regenerate both roots and shoots efficiently, and no transgenic lines could be reliably recovered in subsequent generations. *Agrobacterium* was used to successfully transform cowpea explants (Garcia *et al*., 1986, 1987), and shoots were regenerated from the explants, but not whole plants. Muthukumar and co-workers (1996) attempted to integrate cowpea transformation and regeneration but failed to recover viable seeds from the primary transformants.

Recently, the group in Canberra attempted to establish a regeneration protocol for cowpea. They achieved shoot development, but roots had to be grafted onto these shoots. They reported that they could recover one transgenic shoot per 1000 explants, and the whole process takes almost a whole year (11 months), (NGICA Newsletter Volume 1, No. 4, October 10, 2004; http://www.isp.msu.edu/ crsp/WorkplansforFY05/wa5-a1.pdf).

Most protocols for plant transformation require regeneration of whole plants from leaf or stem explants, calli or single cells, but there are no efficient regeneration methods for legumes. As a result there is no reliable and efficient method for cowpea transformation. Current attempts to develop legume transformation procedures aim at '*in planta*' transformation which excludes tissue culture steps (Hansen & Wright, 1999).

The group at Michigan State University adopted an approach that completely avoids regeneration. DNA was delivered to the apical regions of seedlings by electrophoresis in an attempt to transform cells destined for meiosis (http:/www.isp.msu.edu/ crsp/WorkplansforFY05/wa5-a1.pd). Transgenic sectors developed on leaves, and Southern analysis demonstrated the presence of transgenes in these sectors, but transformation efficiencies need to be improved, and a mechanism for ensuring heritability to subsequent generations need to be developed. It is expected that such procedures will depend on the age of the plant to be transformed and the particular organ targeted for transformation. Reproductive cells and embryonic and meristematic tissues are possible target tissues for transformation leading to heritable transgenes.

3 MATERIALS AND METHODS

3.1.1 Chemicals and reagents

Restriction endonucleases such as *Eco*RI, *Bam*HI and *Not*I, were obtained from Roche Molecular Biochemicals (Mannheim, Germany). Expand reverse transcriptase, Expand Long Template PCR System and dNTPs were also obtained from Roche Molecular Biochemicals (Mannheim, Germany). Other chemicals such as Trizma base, sodium acetate, potassium acetate, etc were obtained from Sigma-Aldrich (UK).

3.1.2 Plants material

Nicotiana benthamiana seeds for use in Zimbabwe were obtained from Prof. Richard Allison at Michigan State University, USA. *Vigna unguiculata* seeds (variety 475/89) were obtained from the Department of Research and Specialist Services (now AREX), Harare, Zimbabwe. *Nicotiana benthamiana*, *N. clevelandii*, *N. glutinosa*, *N. tabaccum* (cv. White Burley), *N. rustica*, *Vigna unguiculata*, *Phaseolus vulgaris*, *Chenopodium amaranticolor*, *Ch. quinoa*, and *Cucumis sativus* for use in host range studies in the Netherlands were supplied by the Department of Virology, Wageningen Agricultural University, The Netherlands. Transgenic *N. benthamiana* plants were maintained in contained facilities at Kutsaga Research Station of the Tobacco Research Board, in Harare.

3.2.0 Virus, characterisation, purification and production of polyclonal antiserum

Unless otherwise stated, standard molecular biology procedures were carried out according to Sambrooks *et al*., 1989.

3.2.1 Host range studies

Nicotiana benthamiana, *N. clevelandii*, *N. glutinosa*, *N. tabacum* (cv. White Burley), *N. rustica*, *Vigna unguiculata*, *Phaseolus vulgaris*, *Chenopodium amaranticolor*, *Ch. quinoa*, and *Cucumis sativus* were sap inoculated with a Zimbabwean isolate of *Cowpea aphid-borne mosaic potyvirus* (CABMV-Z3) and monitored for symptom development. After three weeks, leaves from these test plants were used to sap-inoculate *Ch. amaranticolor*, which had by then been determined to be a good local lesion host plant for CABMV.

3.2.2 Purification of CABMV-Z3

Virus purification was done according to Chavi and co-workers (1997), with modifications. Leaves from *Nicotiana benthamiana*, *N. clevelandii* and *Vigna unguiculata* plants exhibiting leaf distortion and mosaic at about 14 days post inoculation were cut, weighed and macerated in the cold room. The macerated leaf material was homogenized for 2 min in a blender in one, two or three volumes of extraction buffer (0.05 M potassium hydrogen phosphate (K_2HPO_4), 0.01 M ethylene di-amine tetra-acetic acid (EDTA), 1% (w/v) sodium sulphite, pH 7.6, and 5% (v/v) ethanol added just before use). The homogenate was strained through a double layer of cheese cloth that had been boiled in 1 mM EDTA, pH 8.0. The homogenate was centrifuged for 30 min at 10 000 rpm in a Sorvall GSA rotor in polypropylene bottles. The supernatant was carefully collected and Triton X-100 was added to a final concentration of 1% (v/v). The solution was incubated for 1 hr at 4°C with continuous stirring. The virus was pelleted by centrifugation for 4 hr at 4°C on a 20% (w/v) sucrose pad in extraction buffer at 25,000 rpm (72,700 x $g_{max;}$Beckman Ti45 rotor). The supernatant was carefully discarded and about 1 ml of re-suspension buffer (0.005 M potassium hydrogen phosphate, (K_2HPO_4), 0.005 M potassium di-hydrogen phosphate, (KH_2PO_4), pH 7.2) was added to each Ti45 tube containing the pellets. The partially purified virus suspensions were pooled and weighed. Optical grade caesium chloride (Gibco BRL) was

added to the virus suspension to a final concentration of 26.5, 27.5 or 28.5%(w/v). The resulting suspension was dispensed into Ultra-clear tubes and centrifuged at 38,000 rpm (175,475 x g_{max}) in a Beckman SW55 rotor at 4°C for 16 hr. The opalescent band (viewed with a narrow beam of light in a dark cold room) that formed at approximately half a centimetre from the bottom of the Ultra-clear tubes was collected with a syringe fitted with a bent needle and put in a dialysis tube previously boiled in de-mineralised water containing about 1 mM EDTA (pH 8.0) as for the cheese cloth. The virus solution collected was dialysed against three 3-hr changes of a 100-fold excess of the re-suspension buffer at 4°C. For increased purity, the caesium chloride banding was repeated. After dialysis, the virus concentration was determined spectrophotometrically based on the relationship that a 1 mg/ml solution has an extinction coefficient of 2.4 at 260 nm in a light path of 1 cm.

In alternative virus purification procedures the following variations to the basic protocol were performed: addition of Triton X-100 to the extraction buffer and skipping the stirring at 4°C for one hour; use of sodium hydrogen phosphate buffers instead of potassium hydrogen phosphate buffers; addition of (i) 0.01% β-mercaptoethanol (ii) 10 mM $MgCl_2$ (iii) both 0.01% β-mercaptoethanol and 10 mM $MgCl_2$ to both extraction and re-suspension buffers; purification using a 10 – 50% (w/v) sucrose density gradient; purification by polyethylene glycol (PEG) precipitation; and CsCl gradient purification of PEG precipitated virus. The purification products were analysed spectrophotometrically and by sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS-PAGE). The SDS-PAGE gels were run for 40 min at 20 mA, and then for 50 min at 40 mA. The purified virus was used for electron microscopy, production of polyclonal antiserum, and also for viral RNA isolation, especially for experiments to clone the full length CABMV genome.

3.2.3 Electron Microscopy

Leaf squash and purified virus samples were negatively stained with potassium phosphotungstate (PTA) and analysed by electron microscopy. One drop of sample was added to a mounting grid, allowed to stand for 15 sec before excess fluid was gently blotted from one side. One drop of 5% PTA was added to the sample on the grid and gently blotted after 15 sec. The grid was mounted on a Phillips CM or Zeiss EM109 electron microscope and imaged at a magnification of x 12 000.

3.2.4 Production and processing of Antiserum

Virus purified from *N. clevelandii* was used as antigen in the production of rabbit polyclonal antiserum against CABMV. A total of 100 μg of purified CABMV isolate Z3 was diluted in 1.5 ml PBS (0.15 M sodium chloride, 10 mM sodium phosphate, pH 7.2) and thoroughly mixed with an equal volume of Freund's adjuvant and injected into a rabbit intramuscularly. In a booster inoculation 2 weeks later, 500 μg of CABMV-Z3 were diluted into 1.5 ml of PBS and thoroughly mixed with 2 ml of Freund's incomplete adjuvant and injected into the rabbit as before. About 40 ml of blood were bled from the ear of the rabbit at two-week intervals thereafter.

The blood was incubated at room temperature for about 8 hr and serum separated from the blood clot using a filter funnel. The serum was centrifuged at 5 000 rpm (3 024 x g) in a JA 25.50 rotor (Beckman Inc., Fullerton, CA, USA). The supernatant was transferred to a 5 ml screw cap tube and stored at –20°C. Aliquots for immediate use were stored at 4°C.

3.2.5 Determination of the antiserum titre

Three micro litres of 1 in 10 to 1 in 1 000 000 dilutions in PBS of uninfected sap, infected *N. benthamiana* sap and purified virus were separately spotted on blotting membrane and allowed to dry. The blots were incubated overnight in blocking buffer (5% (w/v) elk, 2% (w/v) sucrose) and washed 3 times in PBS-T (0.15 M sodium chloride, 10 mM sodium phosphate, pH 7.2, 0.05% Tween-20). The antiserum was diluted 1000, 2000, 5000 and 10 000 times and incubated with the blots for 2 hr at 37°C. The antiserum was discarded and the blots were washed 3 times with PBS-T. Four microlitres of goat anti-rabbit immunoglobulin conjugated to alkaline phosphatase (Gibco-BRL, Breda, Netherlands) were diluted in 20 ml of PBS-T and incubated with the blots for 2 hr at 37°C. The blots were washed 3 times in PBS-T and then incubated in alkaline phosphatase buffer, AP-9.5 (0.1 M Tris-HCl, pH 9.5, 0.1 M sodium chloride, 50 mM magnesium chloride) for 10 minutes at room temperature on a shaker. The blots were developed by transferring them to 5 ml of AP-9.5 containing 22 µl nitroblue tetrazolium (NBT) reagent (Sigma-Aldrich SA, final concentration 0.40 mM), and 16.2 µl 5-bromo-4-chloro-3-indolyl-phosphate (BCIP, Sigma, final concentration 0.45 mM) for about 10 min. The reaction was stopped by rinsing with water, and washing with PBS-T. The membrane was dried between filter papers. The antiserum was also used to develop semi-dry blots of samples from virus purification experiments.

3.3 Cloning and sequencing of the 3' terminal region of CABMV

3.3.1 Total RNA isolation

In a procedure to isolate total RNA from CABMV-infected plants, 100 mg of CABMV infected *N. benthamiana* leaves were placed in a sterile mortar and chilled at –80°C. The leaf material was ground using a chilled pestle and transferred to a sterile eppendorf tube. Four hundred

microlitres of RNA extraction buffer (760 mM Tris-HCl, pH 8.8, 10 mM LiCl, 0.4 mM EDTA, and 0.1% (w/v) SDS) were added and mixed before 400 µl of phenol were added. The tube was incubated at 65°C for 5 min, with occasional mixing. The tubes were centrifuged at 10 000 rpm for 5 min, and the aqueous phase transferred to a new tube. The aqueous phase was extracted twice with phenol/chloroform. The RNA was precipitated by adding one-tenth volume of 3M sodium acetate (pH 5.2) and two-and-a-half volumes of absolute ethanol, and incubated at –80 °C for at least 30 min. The tubes were centrifuged at 10 000 rpm for 5 min, and the supernatant decanted. The RNA pellet was washed twice with 70% (v/v) ethanol and dried for 10 min under vacuum. The RNA was dissolved in 50 µl DEPC-treated water and used immediately, or stored in absolute ethanol at –80°C for long-term storage. The total RNA was used in RT-PCR to amplify a fragment of the CABMV genome that includes the 3' untranslated region, the CP and part of the replicase genes.

3.3.2 Reverse transcription – polymerase chain reaction (RT-PCR)

To a sterile eppendorf tube 10 µl of total RNA were added and incubated at 65°C for 5 min and immediately placed on ice. The following were then added to the same tube: 8 µl of 5X reverse transcription buffer (250 mM Tris-HCl, 200 mM KCl, 30 mM $MgCl_2$, 50 mM dithioerythritol, pH 8.3), 2 µl of dNTP mix (2.5 mM each of dATP, dCTP, dGTP and dTTP; Gibco BRL), 2 µl of an oligo dT primer (40 ng/µl, sequence: 5'-T_{20} CCTAGG GC-3'), 4 µl of 100 mM DTT (Gibco BRL, Middlesex, England), 1 µl of 40 000 U/µl RNAsin (Promega), 11 µl of DEPC-treated water and 2 µl of 1 U/µl M-MuLV reverse transcriptase (Gibco BRL, Middlesex, England). The reaction was incubated at 37°C for 60 min, and stopped by incubating at 65°C for 10 min, and used for PCR or stored at –20°C.

The PCR was set up as follows. To a tube containing 31 µl sterile distilled water, 5 µl of reverse transcription products, 4 µl of dNTP mix (2.5 mM each of dATP, dCTP, dGTP and dTTP), 5 µl of 10X *Taq* polymerase buffer (0.5 M Tris-HCl, pH 9.0, 0.5 M KCl, 0.07 M $MgCl_2$, 2 mg/ml BSA), 1 µl 25 mM $MgCl_2$, 2 µl of 40 ng/µl primer P89/1747, 2 µl of primer based on a conserved potyviridae replicase sequence (40 ng/µl, sequence: 5'GAC GAA TTC TG(T/C) GA(T/C) GC(T/G/C) GAT GG(T/C) TC-3'), and 2 µl of 0.1 U/µl Super *Taq* DNA polymerase (HT Biotechnology Ltd) were added. The tubes were overlaid with mineral oil, except when thin-walled tubes were used in a thermal cycler with a heated lid. Initial denaturation was carried out at 94°C for 1 min. Amplification was carried out for 36 cycles of melting at 94°C for 1 min, annealing at 53°C for 1 min and extension at 72°C for 2 min, at the end of which the tubes were stored at 4°C in a Thermal Cycler 480 (Perkin Elmer, Cheshire, UK).

The products were run on a 0.8% (w/v) agarose gel to which ethidium bromide was added to a final concentration of 0.05 µg/µl in 0.5X TBE (44.5 mM Tris-borate, pH 8.0, 1 mM EDTA). Low melting point agarose in 1 X TAE (40 mM Tris-acetate, pH 8.3, 1 mM EDTA) under low voltage was used whenever the DNA was to be recovered from gel.

3.3.3 Recovery of DNA fragments from gel by phenol extraction

One of two methods of DNA recovery from gel was used depending on availability of reagents. The piece of gel containing the fragment was cut from the gel, the volume made up to 100 µl with TE. An equal volume of phenol was added, and incubated at 65°C until molten. The tube was then immediately frozen at –20°C for at least 2 hr, and centrifuged at 12 000 rpm for 10 min at room temperature. The solution was extracted once more with phenol, once with phenol/chloroform, and finally with chloroform. One-tenth volume of 3 M sodium acetate and

two volumes of absolute ethanol were added to the aqueous phase, mixed and incubated at –20°C for at least 2 hr. The DNA was pelleted by centrifugation at 12 000 rpm for 10 min, washed twice with 70% ethanol, dried, and resuspended in 20 µl sterile distilled water.

In an alternative procedure, depending on intended use and availability, DNA was recovered from gel using the GIBCO BRL agarose gel extraction kit according to the manufacturer's recommendations (GIBCO BRL, Madison, USA)

3.3.4 Cloning into pGEM-T vector

The recovered 1.2 kb 3' terminal region of the CABMV genome was ligated into pGEM-T vector to give pGEM-CPRep using the pGEM-T cloning kit according to the manufacturer's instructions (Roche Molecular Biochemicals, Germany). The concentration of recovered DNA was estimated by agarose gel electrophoresis, and 7.5 µl (50 ng) were mixed with 0.5 µl of pGEM-T vector (50 µg/ml), 1.0 µl of 10X ligation buffer, and 1.0 µl of T4 DNA ligase, and incubated at 15°C overnight.

The ligated DNA was used to transform competent *E. coli* DH5α cells that were then plated on 2YT agar medium supplemented with 100 mg/l ampicillin, 200 µM IPTG and 20 µg/l X-gal as described in Section 3.3.6. White colonies were picked, grown overnight and used in a procedure to isolate plasmid DNA. The plasmid DNA was characterised by *Pst*I/*Sst*II digestion.

3.3.5 Preparation of competent *E. coli* cells

A fresh LB plate was streaked with *E. coli* from a glycerol stock and incubated at 37°C overnight. An individual colony was picked from the plate and used to inoculate 10 ml of LB medium and incubated in a shaking incubator at 37°C overnight. The following day, 1 ml of the

overnight culture was used to inoculate 100 ml of fresh LB medium and incubated in a shaking incubator at 37°C. The OD of the culture was monitored at 600 nm until it reached 0.8 (almost saturation). The cells were chilled on ice for 10 min and centrifuged at 3 000 rpm for 10 min at 4°C. The pellet was resuspended in half the original volume of ice-cold calcium chloride solution (50 mM $CaCl_2$, 10 mM Tris-Cl, pH 8.0), and incubated on ice for 15 min. The cells were pelleted by centrifugation at 2 500 rpm for 5 min at 4°C. The pellet was resuspended in one-tenth of the original volume of ice-cold calcium chloride solution. Fifty per cent glycerol was added to the cell suspension to a final concentration of 15%. The cells were dispensed in 0.2 ml portions in pre-chilled eppendorf tubes and stored at –80°C.

3.3.6 Transformation of competent *E. coli* cells

A 200 µl aliquot of competent cells was thawed on ice and 2 µl of plasmid DNA (0.5 µg/µl) were added, mixed very gently and incubated on ice for 30 min. The reaction tube was transferred to a 42°C water-bath and incubated for exactly 2 min at the end of which it was quickly transferred back to ice and incubated for another 2 min. To allow cells to recover from the heat shock, 800 µl of 2YT or LB medium pre-warmed to room temperature were added to the tube and incubated at 37°C for 1 hr. After the incubation period 50 µl, 100 µl or 200 µl aliquots were plated on LB or 2YT agar medium containing the appropriate antibiotics. The plates were incubated at 37°C overnight.

3.3.7 Plasmid DNA isolation by the alkaline lysis method

Individual white colonies were picked up from overnight LB agar medium supplemented with 100 mg/l ampicillin, 50 µM IPTG and 20µg/l X-gal, and used to inoculate 10 ml of LB broth supplemented with 100 mg/l ampicillin. The tubes were incubated at 37°C overnight in a shaking

incubator. The overnight culture was transferred to a 1.5 ml eppendorf tube and pelleted by centrifugation at 6 000 rpm for 2 min in a micro-centrifuge. The supernatant was decanted and the pellet resuspended in 200 µl of GTE buffer (50 mM glucose, 25 mM Tris-HCl, pH 8.0, 10 mM EDTA). The cells were lysed by the addition of 400 µl of lysis buffer (0.2 M sodium hydroxide, 1.0% (w/v) SDS), and incubated on ice for 5 min. Protein was precipitated by addition of 300 µl of 3:5 M potassium acetate (3 M potassium, 5 M acetate, pH 5.2), mixed vigorously and incubated on ice for 5 min. The tubes were centrifuged at 12 000 rpm for 5 min, and the supernatant transferred to a new tube. The plasmid DNA was precipitated by the addition of 540 µl of isopropanol and pelleted by centrifugation at 12 000 rpm for 5 min. The DNA pellet was washed twice with 70% ethanol, air-dried for 30 min and resusupended in 50 µl TE-RNase (10 mM Tris-HCl, 1 mM EDTA, 1 mg/ml RNase A).

3.3.8 Restriction endonuclease analysis

To an eppendorf tube containing 10 µg of plasmid DNA in 10 µl of TE buffer, 2 µl of 10 X RE buffer, 6 µl of sterile distilled water and 2 µl of the appropriate restriction endonuclease were added, mixed and incubated at 37°C for 2 hr to overnight. After incubation, 10 µl of the reaction were transferred to a new tube, mixed with 2 µl of 6X gel loading dye (0.25% bromophenol blue, 0.25% xylene cyanol FF, 15% Ficoll Type 400 [Pharmacia, Glyfalda, Greece] in water) and loaded on a 0.8% (w/v) agarose gel. Electrophoresis was carried out for at least 1 hr at 3 - 5V/cm, and the gel was viewed under UV and a photograph taken using a Polaroid camera, Cybertech CSI TV camera or alternatively the image was captured using a Syngene GeneGenius BioImaging System, (Synoptics Ltd, Cambridge, UK).

3.4.1 Expression of CABMV CP in *E. coli*

The cloned 1.2 kb 3' terminal portion of the CABMV genome (section 3.3) was used as template in a PCR utilizing primers that result in amplification of a coat protein gene that is translatable in prokaryotic systems.

The primers were: CABP1ND: 5'GG**C ATA TG**T CTG ATG AAA GAC AAA AGC-3', which introduces an ATG translation start codon, and an *Nde*I site for use in cloning; and CABP2BA: 5'CCG **GGA TCC** TCA CTG CCC ATG CGT CAT-3', which has the translation stop codon, and a *Bam*HI site for use in cloning.

The PCR was set up as follows. To a tube containing 24 µl sterile distilled water, 1 µl of template, 2 µl of dNTP mix (2.5 mM each of dATP, dCTP, dGTP and dTTP), 4 µl of 10 X *Taq* polymerase buffer, 4 µl 25 mM $MgCl_2$, 2 µl of 40 ng/µl primer CABP1ND, 2 µl of 40 ng/µl primer CABP2BA, and 1 µl of 5U/µl *Taq* DNA polymerase (Promega Life Science, Southampton, UK; Madison, WI, USA). The tubes were overlaid with mineral oil. In the first cycle denaturation was carried out at 94°C for 2 min, annealing at 54°C for 1 min, and extension at 72°C for 2 min. In the subsequent 35 cycles melting was done at 94°C for 1 min, annealing at 62°C for 1 min and extension at 72°C for 2 min, at the end of which the tubes were stored at 4°C. The products were run on a 1.0% (w/v) agarose gel in 1X TBE.

The bands were cut out from the low melting point agarose gel, and the DNA recovered using the Glassmax Kit (GIBCO BRL), ligated into pGEM-T vector and used to transform *E. coli* as described earlier. DNA was isolated from pGEM-CP_{nd} transformants using the CTAB miniprep

protocol, and the DNA digested with *Nco*I/*Nsi*I and *Nde*I/*Bam*HI to excise the insert where present.

In an experiment to clone the CP_{nd} gene into the expression vector pT7.7, the insert was excised from pGEM-CP_{nd} using the restriction endonucleases *Nde*I/*Bam*HI; pT7.7 was also digested with *Bam*HI/*Nde*I. The products were run on a 1.0% (w/v) low melting point agarose gel from which the band corresponding to the insert was cut, and the DNA bands separately recovered using the Glassmax kit (Gibco BRL, MD, USA).

A ligation reaction was set up by mixing 3 µl of pT7.7, 5 µl CP insert, 1 µl 10X ligase buffer and 1 µl ligase. The reaction was incubated overnight in a water bath at 16°C. The ligated DNA was used to transform *E. coli* DH5α cells. Plasmid DNA was isolated from transformants following the CTAB miniprep protocol, and the DNA was digested with *Nde*I/*Bam*HI to excise the insert where present. A maxi prep of the selected recombinants was performed and the DNA was used to transform *E. coli* BL21, an expression cell line.

3.4.2 Expression and purification of CABMV-Z3 CP

The *E. coli* BL21 cells transformed with the recombinant plasmid were grown for 6 – 7 hr in a 200 ml liquid culture. The cells were pelleted and resuspended in lysis buffer (1/2X TBS, 1% Triton X-100, 0.1 mg/ml lysozyme). An aliquot was stored at –20°C for analysis by SDS-PAGE. The rest of the sample was lysed by sonication. The lysed cell material was separated into inclusion bodies (pellet) and the rest of the lysed cell material (supernatant) by centrifugation at 30 000 rpm for 30 min. The different samples were analysed by SDS-PAGE to identify the fraction enriched with CABMV-Z3 CP.

3.5 PCR cloning of the various forms of CABMV coat protein genes

3.5.1 Constructs for plant transformation

The primer pairs shown in Table 5 were designed to introduce the consensus sequence for optimum translation in plants, and used to amplify the CABMV isolate 3 (CABMV-Z3) CP gene variants for use in making expressible CP (CP_k), antisense CP (PC), CP with stop codons in all three open reading frames (CP_{stop}) and the core region of CP (CP_{core}). The clone obtained in Section 3.3 (pGEM-CPRep, the 3' terminal region of the CABMV genome) was used as template.

Table 5: Sequences of primers designed to amplify the various forms of the CABMV coat protein gene. RE recognitions sequences are in bold.

Name of primer	Primer Sequence	Targeted Product
1. IS13 (*Bam*HI)	5'-GCG **GGATCC** AACA ATG TCT GAT GAA AGA CAA AAG GAA C-3'	CABMV-Z3 CP expressible sequence
2. IS12 (*Sal*I)	5'-GCG **GTCGAC** TCA CTG CCC ATG CGT CAT CC-3'	
3. PC (*Sal*I)	5'-CCC **GTCGAC** AACA ATG TCT GAT GAA AGA C-3'	Antisense CABMV-Z3 CP
4. CABP2 (*Bam*HI)	5'-CCG **GGATCC** TCA CTG CCC ATG CGT CAT-3'	
5. CP_{stop} (*Bam*HI)	5'-GGC **GGATCC** AACA ATG TCT GAT GAA TGA CTA AAG G-3'	CABMV-Z3 with stop codons in all three reading frames
6. IS12 (*Sal*I)	5'-GCG **GTCGAC** TCA CTG CCC ATG CGT CAT CC-3'	
7.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
8. CP_{core}P2 (*Sal*I)	5'-CCC **GTCGAC** TCA AAG CAT ACC TTG CC-3'	

The PCR was set up as described earlier (Section 3.4.1) except that the 1.2 kb pGEM-CPRep clone was used as template with appropriate primers (Table 5) and *Taq* polymerase were used. The general PCR cycle conditions were as follows: Initial denaturation was at 94°C for 30 sec;

followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 54°C (except for primer pair 7 and 8 that was annealed at 60°C) for 30 sec, and extension at 72°C for 30 sec. A final extension at 72°C was carried out for 5 min before storage at 4°C.

The amplified genes were digested with *Bam*HI and *Sal*I, and ligated into similarly digested plasmid pCa2Nos (Appendix B), which contains a double enhanced *Cauliflower mosaic caulimovirus* (CaMV) 35S (35S) promoter and nopaline synthase (Nos) terminator separated by *Bam*HI and *Sal*I sites. In each case the ligated DNA was used to transform competent *E. coli* DH5α cells. Plasmid DNA was isolated from the transformants using one of the methods below, and screened by restriction endonuclease digestion to identify recombinant pCa2Nos plasmids. The 35S-(modified) CP-Nos fragments were excised from the appropriate recombinant pCa2Nos plasmids by *Hin*dIII digestion and ligated into the unique *Hin*dIII site of the binary plasmid pBI121 (Figure 20). In each case, 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.

3.5.2 Methods for plasmid DNA isolation

The cetyl trimethyl ammonium bromide (CTAB) method was routinely used to isolate plasmid DNA in experiments to screen for recombinants because it is simple and inexpensive. However, plasmid DNA obtained by this method cannot be used in ligation experiments. Therefore, after identification of clones of interest, larger scale plasmid DNA isolations and purifications were carried out using the alkaline lysis method (Section 3.3.7), or a commercial kit (depending on availability) and the DNA was used in subsequent cloning experiments. Where plasmid DNA was for use in sequencing reactions, recommendations of the commercial sequencing facility

were followed. Other methods of plasmid DNA isolation such as the boiling method were used in an attempt to increase DNA yield of large binary plasmids, from *A. tumefaciens* in particular.

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, one of the following methods of plasmid DNA isolation was used. Plasmid DNA isolation was normally by the alkaline lysis method as described earlier (Section 3.3.7). For highly purified plasmid DNA, the DNA obtained by this method was extracted twice with phenol, once with phenol/chloroform and once with chloroform before precipitation with two volumes of absolute ethanol. Plasmid DNA isolation using Wizard Miniprep or Midiprep DNA Purification Systems (Promega, Madison, WI, USA), whenever required, was according to the manufacturer's recommendations.

3.5.2.1 CTAB Plasmid DNA Isolation.

Pelleted cells were resuspended in 300 µl STET (8% (w/v) sucrose, 50 mM Tris-HCl (pH 8.0), 50 mM EDTA, 0.1% (v/v) Triton X-100) and 6 µl of lysozyme (5 mg/ml) and incubated at room temperature for 5 min. The tubes were placed in boiling water for 45 sec, cooled on ice, and centrifuged at 10 000 rpm for 10 min at room temperature. The blob that formed was removed with a pipette tip, and 8 µl of RNase A (20 µg/ml) were added and incubated at 68°C for 20 min. The DNA was precipitated by adding 60 µl of 2.5% (w/v) CTAB in 0.5 M sodium chloride, and incubated for 5 min at room temperature. The DNA was pelleted by centrifugation at 10 000 rpm for 5 min at room temperature, and resuspended in 300 µl of 1.2 M sodium chloride. The DNA was re-precipitated by adding 600 µl of ethanol and incubated at -80°C for 20 min, and pelleted

by centrifugation at 10 000 rpm for 10 min at room temperature. The DNA pellet was washed twice with 70% (v/v) ethanol, dried under vacuum and resuspended in 50 µl RNase A solution.

3.5.2.2 Boiling Method of Plasmid Isolation

The cell pellet obtained as described above was resuspended in 350 µl of STET. Six microlitres of 50 mg/ml lysozyme were added and incubated at room temperature for 5 min. The tubes were placed in boiling water for 40 sec and centrifuged immediately at 14 000 rpm for 5 min. The supernatant was transferred to a new tube and 40 µl of 3M sodium acetate and 420 µl of isopropanol were added and mixed. The DNA was pelleted by centrifugation at 12 000 rpm for 10 min. The DNA pellet was resuspended in 200 µl TE and extracted twice with phenol, once with phenol/chloroform and once with chloroform. Addition of 20 µl of sodium acetate and 500 µl of ethanol precipitated the DNA, and precipitation was maximised by incubation at –80°C for 30 min. The DNA was pelleted by centrifugation at 12 000 rpm for 10 min and washed twice with 70% ethanol, dried and resuspended in 20 µl of TE-RNase A. Two microlitres were digested in 10 µl and run on gel.

Figure 2 below summerises the cloning strategy used to make pBI121-CP_k, pBI121-CP_{stop}, pBI121-PC and pBI121-CP_{core}.

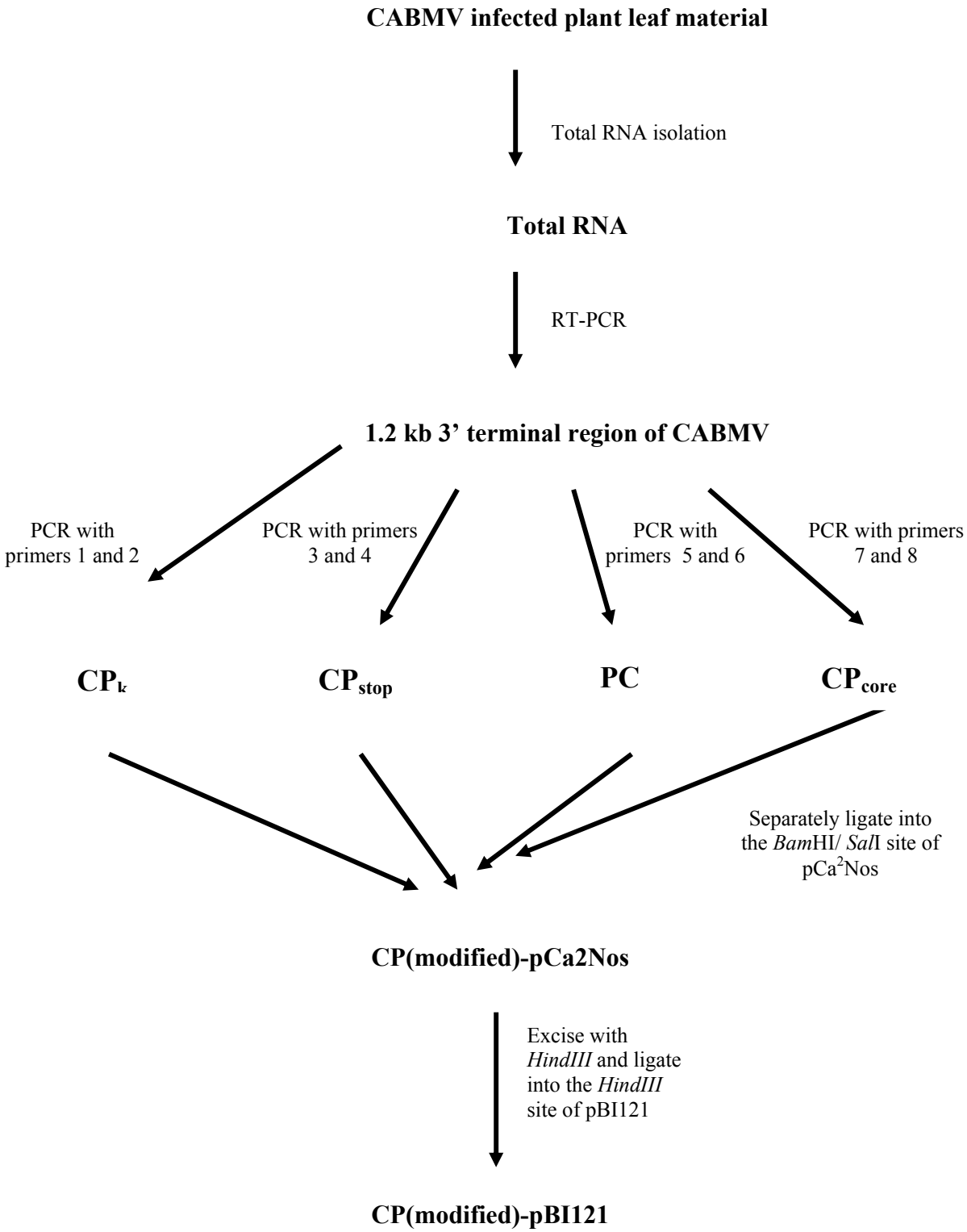

Figure 2: Summary of Cloning Strategy devised to produced binary constructs pBI121-CP_k, pBI121-CP_{stop}, pBI121-PC and pBI121-CP_{core}.

3.5.3 Transformation of *Agrobacterium tumefaciens*

Agrobacterium tumefaciens strain LBA 4404 was grown in 50 ml YEP medium (Appendix E) at 28°C with 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 resuspended in 10 ml of 0.15 M sodium chloride. The cells were pelleted again by centrifugation at 5 000 rpm for 5 min and resuspended in 1 ml of ice-cold 20 mM $CaCl_2$ and stored at –80°C in 200 µl aliquots. One microgram of 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 hr with gentle shaking. The cells were pelleted by centrifugation for 1 min, and resuspended in 100 µl of YEP medium, and plated on YEP or MGL agar (Appendix E) containing 50 mg/l kanamycin and 50 mg/l rifampicin. Plasmid isolations were carried out following the alkaline lysis method (Section 3.3.7) to verify transformants before proceeding to use transformed cells in plant transformation experiments.

3.5.4 Sequencing of CABMV CP constructs

The CABMV CP recombinant 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 the dideoxy dye terminator method in an ABI Prism Model 3100. The CP_{core} primers 1 and 2 described in Table 5 were used.

3.6.0 Cloning of full-length CABMV genome and production of infectious transcripts

The full length CABMV cDNA clone was generated and amplified from a viral RNA template using Expand reverse transcriptase and Expand long template PCR system (Roche Molecular

Biochemicals, Mannheim, Germany) according to the manufacturer's recommendations with modifications as described below. The amplification product was cloned into the Expand Vector I (pEV1, Appendix C), to result in the recombinant vector pEVI-Z3.

3.6.1 Isolation of viral RNA

An amount of 10 µl proteinase K (10 mg/ml solution) was added to 300 µl of purified virus and incubated at 37°C for 30 min. Three hundred microlitres of phenol were added, mixed and centrifuged at 10 000 rpm for 5 min. The preparation was extracted once with an equal volume of phenol, once with phenol/chloroform and finally with chloroform. The viral RNA was precipitated by the addition of one-tenth volume of 3 M sodium acetate and two and half volumes of ethanol, and incubated at –20°C for 2 hr. The viral RNA was pelleted by centrifugation at 10 000 rpm for 10 min, washed twice with 70% (v/v) ethanol, air-dried and re-suspended in 50 µl DEPC-treated water.

3.6.2 Reverse transcription for full length CABMV-Z3 cDNA clones

The key enzyme used in this reaction is Expand Reverse Transcriptase (Roche Molecular Biochemicals, Mannheim, Germany), a version of the Moloney Murine Leukaemia Virus (M-MuLV) reverse transcriptase genetically modified to deplete RNase H activity (Revers *et al*., 1996).

A 57-mer primer (LRP1: 5'-T(21) AGA GAG TTT TGA TAC ACA AAT CTC GGT AGC TAC GCG-3') complementary to the 3' region of the CABMV genome (Mlotshwa *et al*., 2002) was designed and used in a reverse transcription reaction for long cDNA products as follows. To a sterile RNase-free, DNase- free eppendorf tube 8.5 µl viral RNA and 2.0 µl of primer LRP1 were

added, incubated at 65°C for 10 min to denature the RNA, and immediately cooled on ice. The following were added to the same tube: 4.0 µl 5X Expand first strand reverse transcriptase buffer, 2.0 µl 100 mM DTT, 2.0 µl 10 mM dNTP mix (sodium salts), 0.5 µl RNase inhibitor (20 units/µl) and 1.0 µl Expand reverse transcriptase (50 units/µl). The reaction was mixed and incubated at 42°C for 1 hr, and stopped by placing on ice.

3.6.3 PCR amplification of full length CABMV-Z3 cDNA

The Expand Long Template PCR System (Roche Molecular Biochemicals, Mannheim, Germany) was used for the amplification of the full length CABMV-Z3 clone. The system is composed of a mixture of recombinant *Taq* DNA polymerase with high processivity, and *Pwo* DNA polymerase with proofreading ability for high fidelity to result in high amplifications of long DNA molecules.

The cDNA obtained in the Section 3.6.2 above was diluted 50 times and used in a PCR for equally long amplification products, with the primer LRP1 and a second 61-mer primer LRP2 (5'-ATG ATA GTG GAC CTC AAG GAT GGG GTC GAA GAA GCA GCA AAA GTG ATT CAA ATT CAA AGG G-3') corresponding to the 5'-region of the CABMV genome (Sithole-Niang *et al*., 1996). The PCR to amplify the full length CABMV genome was carried out as follows. Working on ice, the following were added to an eppendorf tube marked Master Mix 1: 14.50 µl of sterile distilled water, 2.50 µl of 10 mM dNTP mix, 1.50 µl of 10 µM Primer LRP1, 1.50 µl of 10 µM Primer LRP2 and 5.00 µl of 50 x diluted template DNA. Similarly, a second tube marked Master Mix 2 was set up by adding the following: 18.25 µl of sterile distilled water, 5.00 µl of 10X PCR buffer 3 with $MgCl_2$, 1.00 µl additional 25 mM $MgCl_2$ and 0.75 µl of Expand long template enzyme mix. Master mixes 1 and 2 were mixed in a thin-walled eppendorf

tube, vortexed and placed in a Perkin Elmer GenAmp 2400 thermal cycler, and cycled as described below. Initial denaturation was at 94°C for 2 min. The first 10 cycles were carried out at 94°C for 10 sec; 50°C for 30 sec, 68°C for 8 min; the next 20 cycles were incremental at 94°C for 10 sec, 50°C for 30 sec, 68°C for 8 min (+ 20 sec incremental cycle elongation per cycle). A final elongation was done at 68°C for 8 min, before storage at 4°C. The amplification products were run on a 0.6% (w/v) gel stained with ethedium bromide and photographed.

The 10 kb amplification product, corresponding to the expected full length CABMV genome was recovered from gel and ligated into the cosmid Expand^{TM} Vector 1 (pEV1) and used to transfect *E. coli* DH5α cells. DNA was isolated from the transformants and digested with *Not*I to identify recombinants. The details of this experiment are described below.

3.6.4 Cloning of the CABMV-*Z3* amplicon into pEVI vector

Expand vector I (pEVI, 32.6 kb; Roche Molecular Biochemicals, Germany) is a derivative of cosmid pHC 79 (6.5 kb) that contains a 25.9 kb stuffer fragment from bacteriophage λ. The pEVI can therefore take up an insert of 7.0 – 16.5 kb to restore the cosmid to the size range required for packaging.

3.6.4.1 Polishing of the PCR fragments and 5'-phosphorylation

The 10kb PCR fragment was concentrated using the speed vac (Eppendorf-Netherler-Hinz GmbH, Hamburg, Germany) before being used in the procedure below. The following were mixed in an eppendorf tube: 20.0 µl Expand PCR fragment, 8.0 µl 5X T4 DNA polymerase buffer, 4.0 µl 10X phosphorylation buffer, 1.0 µl T4 DNA polymerase, 1.0 µl T4 polynucleotide kinase, and 6.0 µl sterile distilled water. The reaction was incubated at 37°C for 20 min. The

PCR fragment was purified from the reaction buffer using the High Pure Template Preparation Kit (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer's recommendations.

The purified DNA was run on an agarose gel to check quality and estimate concentration. The DNA was diluted to about 30 ng /µl. The following were added to a sterile reaction tube: 8 µl modified PCR fragment (30 ng/µl), 2 µl of 50 ng/µl Expand Vector I, 10 µl of 2X Ligation Buffer^{PLUS}, and 1 µl of 1U/µl T4 DNA ligase. The reaction was mixed and spun briefly to collect, and incubated at room temperature for 30 min and then at –80°C overnight.

3.6.4.2 *In vitro* packaging of DNA

A tube of packaging extract was thawed at room temperature, and placed on ice as it started to thaw. Five microlitres of ligation reaction mix were added to the thawed packaging extract and mixed by pipetting, avoiding air bubbles. The tube was centrifuged down, and incubated at room temperature for 2 hr. At the end of the incubation period, 300 µl of dilution buffer for SM phages (Roche Molecular Biochemicals, Mannheim, Germany) and 20 µl chloroform were added, mixed carefully and briefly centrifuged to collect.

3.6.4.3 Preparation of *E. coli* DH5α magnesium culture

A loopful of bacteria from a glycerol stock of *E. coli* DH5α was streaked onto an LB-agar plate (without ampicillin), and incubated at 37°C overnight. Twenty millilitres of LB medium containing 10 mM $MgSO_4$ and 0.2% maltose in a 100 ml conical flask was inoculated with a single colony of *E. coli* DH5α cells from the agar plate and incubated with shaking at 37°C for 3

– 4 hr when an OD_{600} of 0.8 was reached. The cells were pelleted by centrifugation at 3 500 rpm for 5 min, and resuspended in 10 mM $MgSO_4$, and the OD of the culture adjusted to 0.8 – 1.0.

3.6.4.4 Infection and plating of *E. coli* DH5α magnesium culture

Two sterile 2 ml tubes A and B were set up as follows: To tube A 50 µl of phage supernatant and 100 µl of *E. coli* DH5α Mg culture were added and carefully mixed. To tube B 200 µl of phage supernatant and 400 µl of *E. coli* DH5α Mg culture were added and carefully mixed. Both reactions were incubated at room temperature for 30 min. To each tube, 1 ml LB medium was added, mixed and incubated at 37°C for 1 hr, with careful mixing every 15 min. The tubes were centrifuged at 13 000 rpm for 3 min, the supernatant discarded and the pellet resuspended in 50 µl fresh LB medium. The cells were plated onto LB plates containing 100 µg/ml ampicillin and incubated overnight at 37°C.

3.6.4.5 Minipreparation and analysis of cosmid clones

Into each of 10 Falcon tubes, 10 ml of LB medium containing 100 µg/ml ampicillin were dispensed and inoculated with cells from individual colonies. The tubes were incubated overnight at 37°C in a shaking incubator. Cosmid DNA (putative pEV-Z3) was then isolated from the culture following the alkaline lysis method for plasmid DNA isolation (Section 3.3.7). The cosmid DNA was digested with restriction endonucleases (*Not*I, *Swa*I, *Sfi*I, *Spe*I) and run on gel.

3.6.5 Subcloning of the CABMV full length clone into pSPT18

The full length CABMV-Z3 insert of pEV-Z3 was excised using *Not*I as described above, recovered from gel and blunted. To a tube containing 10 µl of the CABMV-Z3 insert recovered

from pEV-Z3, 3 µl of sterile distilled water, 4 µl of 5X polymerase buffer, 2 µl of 2.5 mM dNTP mix and 1 µl of T4 DNA polymerase were added, mixed and incubated at 37°C for 1 hr. The blunted reaction was extracted once with phenol, once with phenol chloroform, and once with chloroform. The DNA was precipitated by adding one tenth volumes of 3 M sodium acetate (pH 5.2) and two volumes of absolute ethanol and incubated at –20 °C for at least 2 hr.

Plasmid pSPT18 (3104 bp, Appendix D) was linearised with *Hin*cII: To an eppendorf tube containing 10 µl of plasmid DNA, 6 µl of sterile distilled water, 2 µl of 10X *Hinc*II buffer and 2 µl of *Hin*cII were added, mixed and incubated at room temperature for at least 2 hr. After running a small agarose gel to verify that digestion was complete, the digested DNA was extracted once with phenol, once phenol chloroform, and precipitated by adding one-tenth volume of sodium acetate and two volumes of absolute ethanol.

The ligation reaction was set up in an eppendorf tube by mixing 5 µl of the recovered CABMV-Z3 insert of pEV-Z3 with 4.0 µl of linearised pSPT18, 10.0 µl of 2X ligation buffer and 1.0 µl of T4 DNA ligase. The reaction was incubated for 30 min at room temperature before being transferred to –70°C and incubated overnight. A 10 µl aliquot of the ligated DNA was used to transform competent *E. coli* JM109 cell as described earlier, and the transformed cells plated on 2YT agar medium supplemented with 100 mg/l ampicillin. Colonies were picked and grown in liquid culture and DNA was isolated using the alkaline lysis method. The plasmid DNA was digested with *Hin*dIII, *Eco*RI and *Hin*dIII/*Eco*RI in order to identify and characterise the recombinants.

3.6.6 *In-vitro* transcription and tests for infectivity

Cold *in vitro* transcription was carried out using the SP6/T7 Transcription Kit (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer's recommendations. The SP6 RNA polymerase was used with either pEVI-Z3 linearized with *Swa*I, *Sfi* or pSPT18-Z3 linearized with *Eco*RI. The T7 RNA polymerase was used with pEVI-Z3 linearized with *Spe*I or with pSPT18-Z3 linearized with *Hin*dIII.

The following were mixed in an eppendorf tube: 4 µl pSPT18-Z3 DNA (linearized by an appropriate enzyme), 8 µl dNTP mix (2.5 mM each of ATP, CTP, GTP and UTP), 2 µl 10 x transcription buffer, 4 µl sterile distilled water and 2 µl SP6 or T7 RNA polymerase (Roche Molecular Biochemicals, Mannheim, Germany). The reactions were incubated at 37°C for 1 hr. A 10 µl aliquot of the transcription products were run on gel and another 10 µl were aseptically rubbed into the leaves of *N. bethamiana* and observed for the development of symptoms.

3.7 Tobacco transformation

3.7.1 Transformation of *Nicotiana 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 into sections of about 1.5 cm^2 and wounded several times with a pair of forceps. The sections were placed in a petri dish to which 4 ml of callus induction medium (Murashige & 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 *Agrobacterium tumefaciens* cells carrying various pBI121 constructs grown overnight in MGL medium were added, and co-cultivated for 48 hours at 28°C. The bacterial cells were washed off

in MS medium and the ex-plants dried on filter paper before transfer to callus induction medium together with control uninfected leaf sections, for two weeks. The calli 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 and produce seeds.

3.7.2 Screening of R1 generation

First generation generants (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.

3.7.3 Plant genomic DNA isolation

Genomic DNA was isolated from *N. benthamiana* plants either following the SDS method or using the Plant DNA Isolation Kit (Roche Molecular Biochemicals, Mannheim, Germany) depending on availability.

3.7.3.1 Grinding of samples

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 small 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.

3.7.3.2 DNA isolation using the Plant DNA Isolation Kit

To about 100 mg of tissue homogenized in 450 µl of Buffer 1 (resuspension buffer) at room temperature, 30 µl of Buffer 2 (lysis buffer) were added and mixed thoroughly by vigorous shaking, and incubated at 65°C to complete the lysis. To remove proteins, 150 µl of Buffer 3 were added and thoroughly mixed by vigorous shaking and incubated on ice for 5 min. The tubes were centrifuged at 12 000 rpm for 10 min, and the supernatant transferred through a filter to a new tube containing 600 µl ice-cold isopropanol. The supernatant and isopropanol were mixed by gently inverting several times, and incubated at – 20°C for 10 min. The DNA was pelleted by centrifugation at 12 000 rpm for 10 min, the supernatant discarded, and the DNA pellet allowed to air dry for 10 min at room temperature.

The DNA was resuspended by adding 330 µl of Buffer 4 (Resuspension Buffer or TE), swirled gently and incubated at room temperature for up to 30 min with occasional swirling. An equal

volume of phenol was added, mixed gently and the tubes centrifuged at 10 000 rpm for 5 min to separate the phases. The upper aqueous phase was transferred to a new tube, and similarly extracted once again with an equal volume of phenol/chloroform, and once with chloroform. The DNA was re-precipitated by adding 35 µl of 3 M sodium acetate (pH 5.2) and 500 µl isopropanol, and completely mixed by inversion. The tubes were centrifuged at 10 000 rpm for 5 min, the supernatant decanted and the DNA pellet dried in a vacuum drier for 15 min. The DNA was resuspended in 60 µl TE and stored at 4°C.

3.7.3.3 Plant genomic DNA isolation using the SDS method

This procedure is a modification of the method of plant DNA extraction first described by Dellarporta *et al*., (1983). About 0.5 g of *N. benthamiana* leaves were weighed and frozen in a sterile pestle and mortar using liquid nitrogen or stored at –80°C for at least 30 min and ground using the frozen pestle and mortar. The powdered tissue was collected in an eppendorf tube and 500 µl of SDS extraction buffer (0.1 M Tris-HCl (pH 8.0), 2 mM EDTA, 0.5 M NaCl, 1.0% (w/v) Polyvinyl pyrrolidone, 1.7% (w/v) SDS) preheated to 65°C were added, mixed and incubated in a 65°C water bath for 10 min. After 10 min, 160 µl of 5 M potassium acetate were added and mixed. An equal volume of chloroform was added to the tube and mixed. The tubes were centrifuged at 10 000 rpm for 5 min. The aqueous phase was collected in a new tube, and the extraction step repeated. DNA was precipitated by adding an equal volume of isopropanol to the aqueous phase in a new tube, mixed gently by inversion and pelleted by centrifugation the tube at 10 000 rpm for 10 min. The supernatant was carefully aspirated without disturbing the pellet that was then air-dried for 10 min at room temperature. The DNA pellet was dissolved in 400 µl TE-RNAse (10 mM Tris-HCl, 1 mM EDTA, 1 µg/ml RNase A) and incubated for 30 min

at room temperature. At the end of the incubation period, 2 µl of proteinase K (20 mg/ml) were added and incubated at room temperature for 30 min.

An equal volume of phenol was added and mixed for 5 min at room temperature. The tubes were centrifuged at 10 000 rpm for 5 min, and the aqueous phase transferred to a new tube, and similarly extracted once with a 1:1 mexture of phenol:chloroform and once with chloroform. The aqueous phase was transferred to a new tube to which 1/10 volume of 3 M sodium acetate (pH 5.2) and 2.0 volume of chilled absolute ethanol were added and mixed by inversion. The tubes were incubated at -80°C for 30 min, and the DNA pelleted by centrifugation at 10 000 rpm for 10 min, and washed twice with 70% (v/v) ethanol. The DNA was dried and resuspended in 500 µl sterile water.

3.7.4 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 ng/µl), 2.0 µl primer $CP_{core}P1$, 2.0 µl primer $CP_{core}P2$, 5.0 µl 10X 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). The cycling 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.

In alternative amplification reactions, primers CP1 and CP2 were used.

3.7.5 Southern blotting

Genomic DNA of putative transgenic lines was digested with *Eco*RI, *Hin*dIII and *Xba*I 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.

The gel was incubated in two changes of 0.25 N hydrochloric acid for 10 min to depurinate the DNA. The gel was then incubated in two changes of denaturation buffer (1.5 M sodium chloride, 0.5 M sodium hydroxide) for 45 min on a rocking platform. The gel was rinsed briefly in distilled water and then transferred to neutralization solution (1 M Tris-HCl, pH 7.4, 1.5 M sodium chloride) for 30 min on a rocking platform. The gel was placed upside down a large piece of cling wrap on a solid platform. A piece of nylon membrane was cut to exactly the same size as the gel, wetted in neutralization buffer and placed on top of the gel. Three pieces of Whatmann 3MM paper were also cut to the same size as the gel and placed on top of the nylon membrane, and further staked with blotting paper and multi-wipe. A glass plate and a weight were placed on top of the stake. Figure 3 below illustrates the stacking. The staked gel was then placed in a large plastic bag to prevent evaporation, and the DNA allowed to transfer overnight.

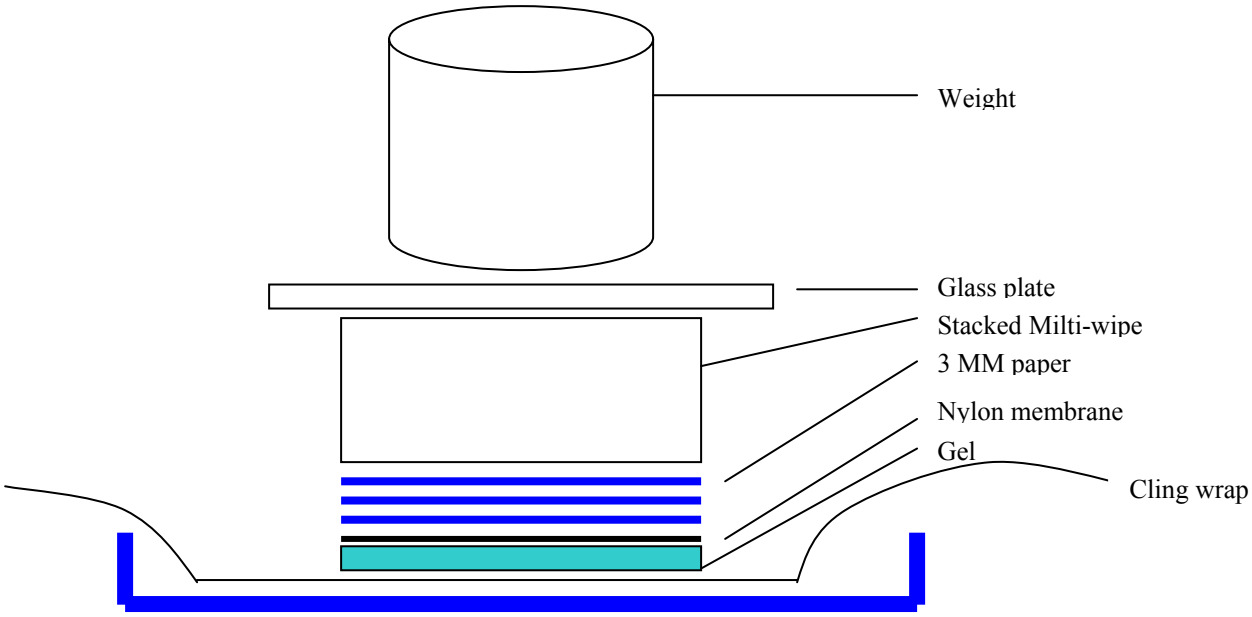

Figure 3: An illustration of stacking for Southern blotting

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 hr.

3.7.6 Hybridisation and Detection of transgenes by the DIG chemiluminescent procedure

3.7.6.1 Probe preparation and labelling

Clone pCa2Nos-CP or pCa2Nos-CP_{core} was digested with *Hin*dIII or *Bam*HI/*Sal*I. 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 denatured at 94°C for 5 min and immediately placed on ice. Four microlitres of DIG High Prime Labelling Mix (Roche Molecular Biochemicals, Mannheim, Germany) were added,

mixed and the reaction incubated at 37°C overnight. The labelled probe was denatured in boiling water for 5 min and immediately placed on ice, ready for addition to hybridisation solution.

3.7.6.2 Hybridisation

The nylon membrane to which the genomic DNA had been fixed was pre-soaked in 2X SSC, before being transferred to a hybridisation bottle to which pre-hybridisation buffer (Roche Molecular Biochemicals, Mannheim, Germany) was then added at a rate of 20 ml per 100 cm^2 of membrane. The tube was incubated at 68°C for 30 min in a rotating hybridisation oven. The pre-hybridisation solution was discarded and replaced with 2.5 ml/100 cm^2 hybridisation solution (pre-hybridisation buffer to which 5 - 25 ng/ml of labelled probe were added per ml of solution). The tube was incubated overnight at 68°C in a rotating hybridisation oven. The membrane was washed twice for 5 min in 2X SSC, 0.1% (w/v) SDS at room temperature, and twice for 15 min at 68°C, in 0.1X SSC, 0.1 (w/v) SDS.

3.7.6.3 Detection of signal

The hybridised membrane was blocked for 3 min in 1 x blocking buffer (10 x blocking stock solution diluted with maleic acid buffer, Roche Molecular Biochemicals, Mannheim, Germany), and washed twice with wash buffer (0.1 maleic acid, 0.15 M sodium chloride, pH 7.5, 0.3% (v/v) Tween 20). The membrane was then incubated with anti-DIG-alkaline phosphatase conjugate. 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.

3.8 Virus Challenge Experiments

After 3 weeks in the greenhouse the T1 plants were about 15 cm tall and were challenged with viral RNA, purified virions or 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.

3.9 Cowpea transformation

The experiments described in this section were to evaluate a novel method for cowpea transformation developed by Prof. R. Allison of Michigan State University which attempts to evade the need for regeneration (NGICA Newsletter Volume 1, No. 3, August 18, 2004; http:/www.isp.msu.edu/ crsp/WorkplansforFY05/ wa5-a1.pdf). The DNA is delivered to the meristematic cells of young seedlings, in the hope that the seedlings develop into chimeric plants with some transformed branches. Transgenic branches will bear seeds with embryos that may carry the transgenes.

3.9.1 Procedure for cowpea transformation by electrophoresis

Cowpea (*Vigna unguiculata* variety 475/89) seeds were sterilized by shaking in 10% (v/v) bleach for 10 min at room temperature, and washed with double distilled water for 5 min. The seeds were then rolled on a moistened paper towel and placed in a beaker with water and incubated in the growth room at 28˚C until the seeds germinated (7 – 12 d).

For each transformation attempt, a seedling was removed from the paper towel, pre-treated (where applicable, according to Table 6) and placed in the transformation tube. About 1 µl of DNA (0.5µg/µl) was mixed with about 9 µl of 2% (v/v) low melting point agarose (made up in transformation buffer) and allowed to set at the tip of a 200 µl pipette whose tip had been widened by cutting. Both the pipette tip and the transformation tube (Figure 4) were filled with transformation buffer (0.12 M LiCl, 1 mM Hepes, 0.54 mM $MgCl_2$, 0.005% L-ascorbic acid, pH 7.2). The setup (Figure 4) was connected to a power source (Figure 4) and allowed to run under current and voltage settings specified in the Table 6 overleaf.

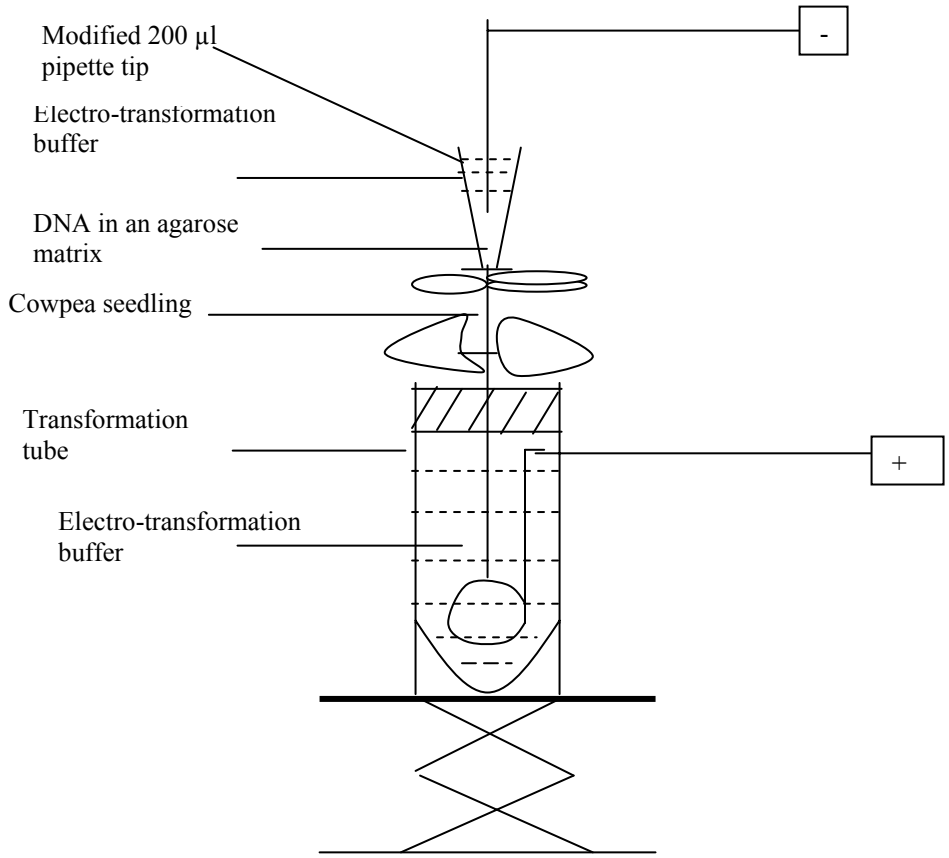

Figure 4: Diagrammatic illustration of the electro-transformation equipment and experimental set-up.

The date of germination, date of manipulation, length of the seedling, pre-treatment, voltage and current applied, distance between electrodes, and duration of electrophoresis were recorded.

3.9.2 DNA constructs used for cowpea transformation

All the binary constructs described earlier were used in this experiment: pBI121-CP_k, pBI121-CP_{stop} and pBI121-PC. Table 6 below summarizes the experiments that were carried.

Table 6: Summary of cowpea transformation experiments

Expt	Construct	Voltage (V)	Current (mA)	Time (min)	Treatment	No. of Plants
Pre-A – circular plasmid	pBI121-CP_k pBI121-CP_{stop} pBI121-PC pBI121-CP_{core} pBI121	125	0.15	15	Every third experiment was AC	9 plants per construct
Plasmids linearised with *Nhe*I						
A	pBI121-CP_k	125	0.15	15	-	100
B	pBI121-CP_k	125	0.15	15	Punched meristems	100
C	pBI121-CP_k	125	0.15	15	A: -	50
A/C 30 sec					B: punched meristems	50
D	pBI121-CP_k	250	0.15	15	Increased voltage	100
E	pBI121-CP_k	125	0.15	15	Manipulated at > 30°C	100
F	pBI121-CP_k	125 or	0.15	15	D: 250 V	50
A/C 30 sec		250			E: >30°C pre-treatment	50
Plasmids digested with *Nde*/ *Nhe*1						
G	pBI121-CP_k	125	0.15	15	Nhe I/ Nde I double digests	100
H	pBI121-CP_k	125/250	0.15	15	Some 250V, others 125V	100
AC 30 sec	pBI121-CP_k	30/40V	0.15/1.0	15	G: 125V	50
					H: 125/250V	50
J	pBI121-CP_k	125	0.15	15	Meristems and leaves pre-treated with 0.1 M HCl	50
I	pBI121-CP_k	125	0.15	15	Meristems and leaves pretreated with 0.1 M $CaCl_2$	50
K	pBI121-CP_k	125	0.15	15	Seedlings exposed to >35°C for 1hr before manipulation	50
L	pBI121-CP_k	30	1.0	15	J: HCl	25
AC 30 sec					I: $CaCl_2$	25
					K: >35°C	25
M	CP_{stop}-pBI	125	0.15	15	Heat pre-treatment	50
N	pBI121-CP_k	125	0.15	15	Sprayed with (2) 2,4-D + (0.04) kin after manipulation	50
O	pBI121-CP_k	30	1.0	15	M: > 35°C, CPstop	25
AC 30 sec					N: 2,4-D/kin CPk	25
P	pBI121-PC	125	0.15	15	Sprayed with (2) NAA + (1) BAP	50
Q	pBI121-CP_{core}	125	0.15	15	Exposed to > 35°C before manipulation, and spray with (2) 2,4-D + (0.004) kin after tran	50
R	P:pBI121-$CP_{antisense}$	30	1.0	15	Sprayed with P:NAA/BAP	25
AC 30 sec	Q: pBI121-CP_{core}				Q: > 35°C and 2,4-D/kin	

After treatment, the seedlings were briefly rinsed with sterile distilled water and planted in pots, and transferred to the greenhouse. The plants were allowed to grow to maturity, and seeds were harvested and stored in envelopes, each envelope representing a manipulated plant.

3.9.3 Spectrophotometric/visual GUS Assays

At least 50% of the seeds in each envelope were germinated separately in seedling trays in the greenhouse. At the 10 – 14 day stage, each seedling was sampled for GUS assay as described below. Leaf material was collected by punching a leaf disk of about 0.04 mg into an eppendorf tube, and 500 µl of Extraction Buffer (50 mM sodium phosphate, pH 7.0, 10 mM Na_2EDTA, 0.1% Triton X-100, 0.1% sodium lauryl sarcosine/sarcosinate (Sarkosyl), and 10 mM β-mercaptoethanol) were added, and ground using a grinder fitted with a micro-pestle. The tubes were centrifuged at 12 000 rpm for 15 min in a micro-centrifuge, and 100 µl of the supernatant were transferred to a new eppendorf tube. Nine hundred microlitres of Detection Buffer (50 mM sodium phosphate, pH 7.0, 10 mM β-mercaptoethanol, 1 mM p-nitrophenyl glucuronide (X-gluc), 0.1% Triton X-100) were added, mixed and the tubes were incubated at 37°C overnight. The reaction was stopped by the addition of 400 µl of 2.5 M 2-amino-2-methyl propanediol. The tubes were scored for the presence of GUS activity by visual inspection and by spectrophotometry. GUS positive samples appear blue, and activity can be quantified by absorbance at 415 nm.

Genomic DNA was also isolated from putative transgenic plants using the Plant DNA Isolation kit (Roche Molecular Biochemicals, Mannheim, Germany). The DNA was used in PCR to detect the GUS and CP genes as described earlier.

4 RESULTS

4.1 Host Range Studies

The CABMV was inoculated into *Nicotiana benthamiana*, *N. clevelandii*, *N. glutinosa*, *N. tabacum* (cv. White Burley), *N. rustica*, *Vigna unguiculata*, *Phaseolus vulgaris*, *Chenopodium amaranticolor*, *Ch. quinoa*, and *Cucumis sativus* plants but could only be passed to the indicator *Ch. amaranticolor* plants from *V. unguiculata*, *N. benthamiana*, *N. clevelandii* and *Ch. quinoa*. This shows that these four plants species are possible hosts of the virus. The symptoms observed were as follows: (i) *N. benthamiana*: Dark green vein banding, mosaic, and leaf distortion (Figure 6). Vein clearing also occurred in the early stages of infection in young leaves. (ii) *N. clevelandii*: Mild chlorotic spots, otherwise the plants appeared healthy. (iii) *V. unguiculata*: Dark green vein banding, mosaic and leaf distortion. Medium sized chlorotic spots were observed on older leaves. (iv) *Ch. quinoa*: General leaf necrosis. (v) *Ch. amaranticolor* : Chlorotic spots were observed on inoculated leaves. *Ch. amaranticolor* is therefore a good local lesion host for the virus, and was used in tests to identify the fractions with virus during virus purification.

Figure 5: Photograph of *Nicotiana benthamiana* leaves showing systemic symptoms of CABMV infection (right) contrasted with the appearance of a healthy leaf (left).

Under the test conditions, *N. glutinosa*, *N. tabacum* (cv. White Burley), *N. rustica*, *Phaseolus vulgaris*, and *C. savitus* were shown to be non-hosts of CABMV.

4.2 Virus purification and polyclonal antibody production

Virus purification was attempted from *V. unguiculata*, *N. benthamiana* and *N. clevelandii* under various conditions and the purified virus was analysed spectrophotometrically by scanning in the range 220 - 320 nm. A single peak was obtained at about 270 nm and the A_{260}:A_{280} ratios were around 1.2 as expected for purified potyvirus (Lima *et al*., 1979). Virus concentration was estimated based on the extinction coefficient of 2.4 (mg/ml, 1 cm light path) at 260 nm (Bashir & Hampton, 1995). The highest yield of 25.1 mg of purified virus per kilogram of infected leaf material was obtained from infected *N. clevelandii* using potassium hydrogen phosphate buffers. At the homogenisation step, use of 3 volumes of buffer for every gram of leaf material was best. Mass of leaf material to volume of buffer ratios of 1:1 and 1:2 resulted in loss of virus trapped in the pellet of cell debris at the clarification step. Addition of Triton X-100 after low speed centrifugation gives the highest yields. A 28.5% (w/v) CsCl density gradient was shown to form the best purification gradient since the virus band forms not too close (about 1 cm) to the base of the ultra-clear centrifuge tube, facilitating band recovery.

Virus purification from *N. benthamiana* using sodium hydrogen phosphate buffers resulted in yields of 24.8 mg/kg, which are higher yields than the 17.6 mg/kg when potassium hydrogen phosphate buffers were used. However, lower yields were obtained from *V. unguiculata* using sodium hydrogen phosphate buffers (2.5 mg/kg) compared to potassium hydrogen phosphate buffers (24.0 mg/kg).

Addition of β-mercaptoethanol and 10 mM magnesium chloride to extraction buffer, re-supension buffer or both, and 0.001 mM EDTA to re-suspension buffer was shown to improve the purification yield. β-mercaptoethanol resulted in yields of 17.6 mg/kg, which was higher than the 16.4 mg/kg for $MgCl_2$. There was no significant improvement when both β-mecaptoethanol and magnesium chloride were used together. Degradation as observed on gel was probably less in storage in the presence of β-mercaptoethanol and EDTA (Figure 6). Only β-mercaptoethanol is therefore recommended for both extraction buffer and re-suspension buffer.

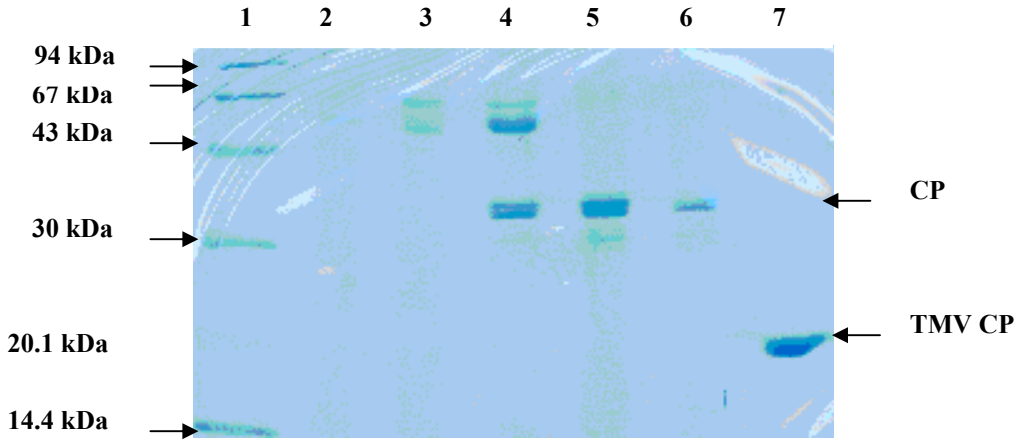

Figure 6: Photographs of a 20% (w/v) SDS-PAGE gel stained with Coomassie Brilliant Blue dye showing proteins in 20 µl of purified virus samples. Isolations were from *N. benthamiana* unless otherwise stated. The lanes are: **1** – Molecular weight marker, **2** – virus purified from *N. benthamiana* using a 1:1 (w/v) ratio of plant material to extraction buffer, **3** - virus purified from *N. benthamiana* using a 1:2 (w/v) ratio of plant material to extraction buffer, **4** - virus purified from *N. benthamiana* using a 1:3 (w/v) ratio of plant material to extraction buffer, **5** - virus purified by separation on $CsSO_4$ density gradient twice, **6** – virus purified by separation on CsCl density gradient twice, **7** – purified TMV for comparison. A single band is seen in lane 6 while lane 5 shows the same band but with a lot of degradation products.

The main protein band in lane 6 was estimated by comparison to molecular weight markers in SDS-PAGE, to be about 32 kDa.

The highest yields obtained by a 10 – 50% (w/v) sucrose gradient purification of clarified sap was 20.6 mg/kg. The virus band occurred at a relative position of 0.4 from the bottom of the tube. However, the virus obtained was very dilute, and most of the virus was lost during the concentration step. The PEG - precipitated virus had a lot of impurities but could be purified to comparable purity levels by using sucrose pad partial purification and CsCl density gradient centrifugation. Only as little as 6.9 mg/kg of infected leaves was obtained using the PEG precipitation method. In all the methods involving two cesium chloride density gradient centrifugation, almost 50% of the virus was lost between successive centrifugations, but purity as indicated by A_{260}:A_{280} ratio, improved to 1.2. Potyvirus-like filamentous particles about 750 nm long were observed using the electron microscope (Figure 7).

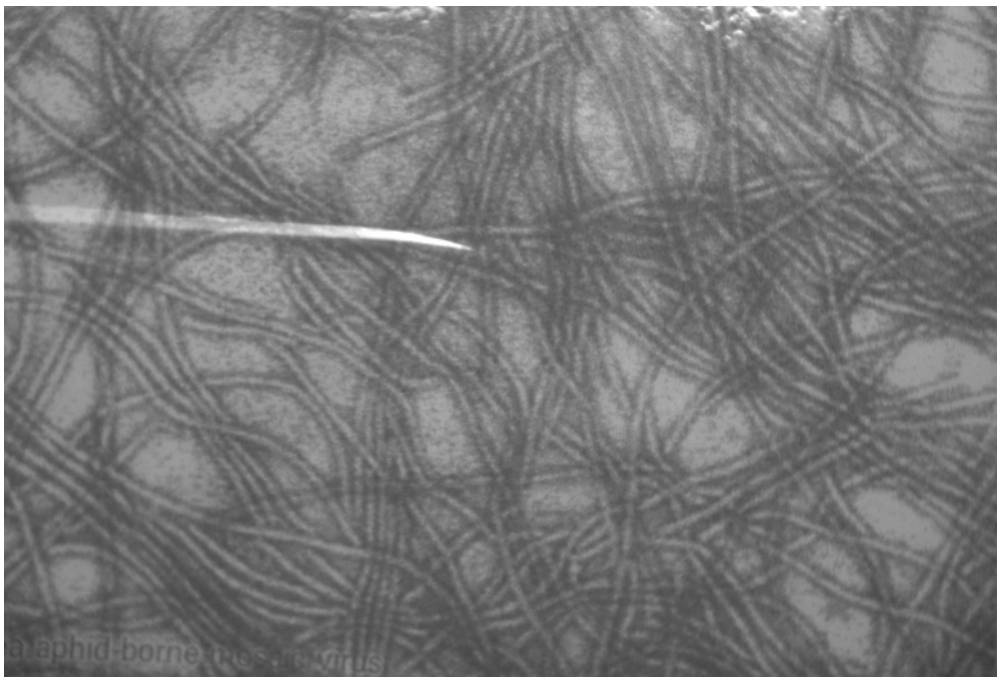

Figure 7: Electron-micrograph of purified CABMV showing a high concentration of filamentous virus particles and little or no impurities. (Magnification: x 12 000)

The purified virus could be passed back to *Ch. amaranticolor*, *N. benthamiana* and *V. unguiculata*, and the same symptoms described earlier were observed. The purified virus was used to raise antiserum against CABMV, in a rabbit. The antiserum was used in an immuno-dotblot assay using uninfected sap, sap from infected plants and purified virus. As little as 46 ng/ml of purified virus could be detected using the polyclonal antiserum (Table 7).

Table 7: Evaluation of polyclonal antisera against purified CABMV.

	Antibody Dilution												
Sample dilution	1.0×10^{-4}			**5.0×10^{-3}**			2.0×10^{-3}			1.0×10^{-3}			
	H	IL	PV	**H**	**IL**	**PV**	H	IL	PV	H	IL	PV	
$1x10^{-1}$	-	+	+	**-**	**+**	**+**	-	+	+	-	+	+	**1st**
$1x10^{-2}$	-	+	+	**-**	**+**	**+**	-	+	+	-	+	+	**Bleed**
$1x10^{-3}$	-	+	+	**-**	**+**	**+**	-	+	+	-	+	+	
$1x10^{-4}$	-	-	-	**-**	**-**	**+**	-	-	-	-	-	+	
$1x10^{-5}$	-	-	-	**-**	**-**	**+**	-	-	-	-	-	+	
$1x10^{-6}$	-	-	-	**-**	**-**	**-**	-	-	-	-	-	-	
$1x10^{-1}$	-	+	+	**-**	**+**	**+**	-	+	+	-/+	+	+	**2nd**
$1x10^{-2}$	-	+	+	**-**	**+**	**+**	-	+	+	-/+	+	+	**Bleed**
$1x10^{-3}$	-	+	+	**-**	**+**	**+**	-	+	+	-	+	+	
$1x10^{-4}$	-	+	+	**-**	**+**	**+**	-	-	+	-	-	+	
$1x10^{-5}$	-	-	+	**-**	**-**	**+**	-	-	+	-	-	+	
$1x10^{-6}$	-	-	-	**-**	**-**	**-**	-	-	-	-	-	-	

Key: H– uninfected leaf, IL – infected leaf, PV– purified virus
-: no reaction, +: positive reaction

Table 7 above shows that antiserum dilution of 5.0×10^{-3} is the best dilution at which to use the antiserum even though it is usable at twice that dilution. More concentrated antiserum may result in false positives. Since the initial purified virus concentration is 4.6 mg/ml, and was detected at a dilution of 1.0×10^{-5}, the detection limit of the antiserum corresponds to 46 ng/ml.

The semi-dry blot developed with the polyclonal antiserum against the purified virus reacted with the protein band at about 32 kDa (Figure 8), thereby confirming the band to be the CP.

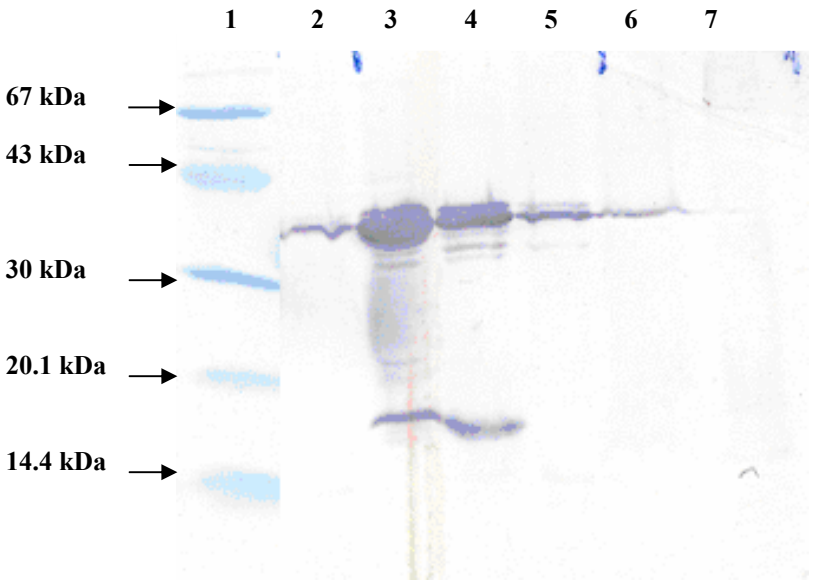

Figure 8: A semi-dry blot of products of various virus purification attempts developed with the polyclonal antiserum raised against CABMV purified from *N. clevelandii*. The lanes are: **1** – Low molecular weight marker, **2** – PEG precipitate further purified by CsCl density centrifugation once, **3** – Sucrose pad pellet further purified by CsCl density gradient centrifugation twice, **4** – PEG-precipitated virus further purified twice by CsCl density centrifugation, **5** – PEG precipitate further purified twice by CsCl density centrifugation, **6** – Sap from a CABMV-Z3 infected *N. benthamiana* plant, **7** – Sap from an uninfected *N. benthamiana* plant.

The gel shows no reaction between the antiserum and the sap from an uninfected *N. benthamiana* plant, while sap from CABMV infected plant shows one distinct band with the same electrophoretic mobility as the band from purified virus. Some purified virus sampleshowever has an additional band at about 18 kDa.

4.3 Cloning and sequencing of the 3' terminal region of CABMV

Total RNA from a CABMV infected *N. benthamiana* plant was used as template in an RT-PCR reaction to amplify the 3' terminal region of the virus. Figure 9 below shows that the expected 1.2 kb fragment was amplified.

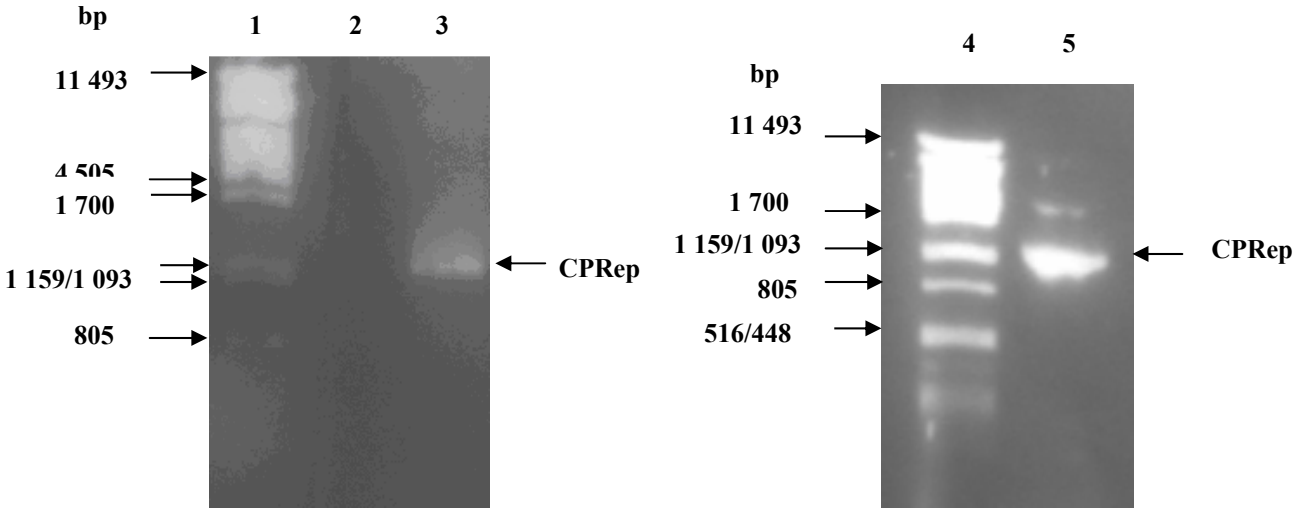

Figure 9: Photograph of a 0.8% (w/v) gel showing the 1.2 kb PCR amplification product corresponding to the 3' terminal region of CABMV. The lanes are: **1** and **4** – molecular weight marker (λ-*Pst*I digest, bp), **2** – water control, **3** – the 1.2 kb PCR amplification product with CABMV infected *N. benthamiana* as template, **5** – the 1.2 kb amplification product recovered from gel.

The 1.2 kb amplification product (CPRep) was recovered from the gel (Figure 9, lane 5) and ligated into pGEM-T vector and used to transform competent *E. coli* DH5α cells. DNA was isolated from the putative recombinants, and screened by *Pst*I/*Sst*I digestion (Figure 10).

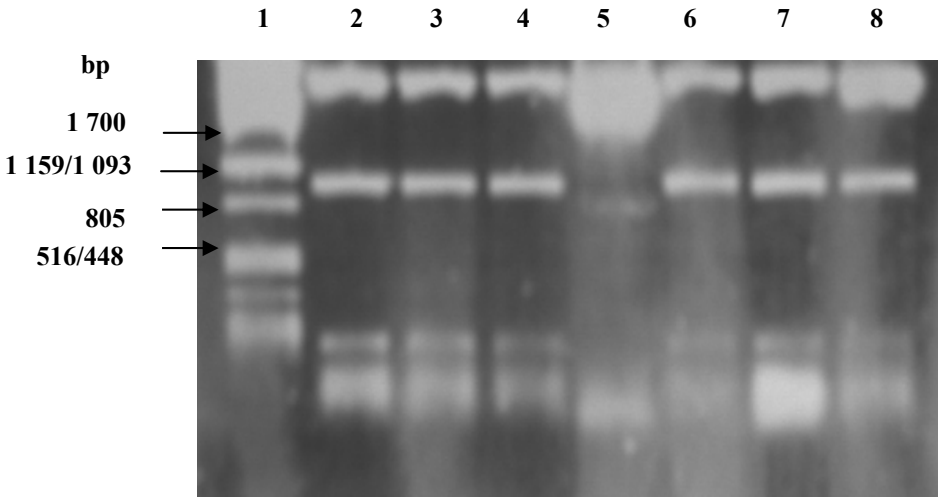

Figure 10: Photograph of a 0.8% agarose gel of plasmid DNA isolated from *E. coli* transformants and digested with *Pst*I/*Sst*I in an experiment to identify recombinant pGEM-T clones with 1.2 kb CABMV 3' terminal region insert. The lanes were loaded as follows: **1** – molecular weight marker (λ-*Pst*I digest, kb), **2** to **8** – putative recombinants. Only lane 5 has an empty vector.

The purified CPRep DNA insert was sequenced, and the sequence obtained is shown in Figure 11.

```
   1  GAA TTC AAG GAA TTA GCT AGT CTT GGA AAA GCT CCT TAT ATT GCG GAA ACT GCT CTT AAA      60
   1   E   F   K   E   L   A   S   L   G   K   A   P   Y   I   A   E   T   A   L   K      20

  61  AAG CTT TAC ACT GAT GAG CAG GCA TCA GAG AAG GAA TTG CAG AGA TAT CTT CAA GAT ATC     120
  21   K   L   Y   T   D   E   Q   A   S   E   K   E   L   Q   R   Y   L   Q   D   I      40

 121  CTT TCC TTC TAC GAT GAT AGC GAA TCA GAG GAT GTT GTG CTC CAA TCT GAT GAA AGA CAA     180
  41   L   S   F   Y   D   D   S   E   S   E   D   V   V   L   Q   S   D   E   R   Q      60

 181  AAG GAA CTG GAT GCA GGT AAG GAC AAA GAC AAG GCT AAG GAA GCT AGA GAG CAA TCA ACG     240
  61   K   E   L   D   A   G   K   D   K   D   K   A   K   E   A   R   E   Q   S   T      80

 241  CAA CAG AAG CAA GCA AAG AAT AAA GGG GCC AAG GAA ACA GAA AGA GAT GTA GCA GCT AGT     300
  81   Q   Q   K   Q   A   K   N   K   G   A   K   E   T   E   R   D   V   A   A   S     100

 301  TCT TCA GGG CAA CTA GTC CCA CGC CTG CAG AAG ATT AGC AAA AAG ATG AAT CTT CCT ATG     360
 101   S   S   G   Q   L   V   P   R   L   Q   K   I   S   K   K   M   N   L   P   M     120

 361  GTC GCT GGT AGG CTT ATC CTT AAT ATT GAT CAT TTG ATA GAA TAT AAG CCA AAA CAG ATT     420
 121   V   A   G   R   L   I   L   N   I   D   H   L   I   E   Y   K   P   K   Q   I     140

 421  GAT CTG TAT AAC ACC AGA GCA TCA AAG GCA CAG TTC AAC ACA TGG TTT GAG GCC GTC AAG     480
 141   D   L   Y   N   T   R   A   S   K   A   Q   F   N   T   W   F   E   A   V   K     160

 481  GAG GAG TAT GAG CTG GAT GAC GAC AAG ATG AGT GTA ATT ATG AAT GGT TTC ATG GTA TGG     540
 161   E   E   Y   E   L   D   D   D   K   M   S   V   I   M   N   G   F   M   V   W     180

 541  TGC ATT GAA AAT GGA ACC TCA CCT GAT GTG AAT GGA GTG TGG ACT ATG ATG GAT GGA GAT     600
 181   C   I   E   N   G   T   S   P   D   V   N   G   V   W   T   M   M   D   G   D     200

 601  GAG CAA GTG GAA TTT CCG CTT AAA CCC ATT GTG GAA AAC GCA AAA CCC ACA CTC AGA CAA     660
 201   E   Q   V   E   F   P   L   K   P   I   V   E   N   A   K   P   T   L   R   Q     220

 661  GTT ATG CAC CAT TTC TCA GAC GCA GCT GAA GCG TAC ATT GAG ATG AGA AAT TCC GAA GGG     720
 221   V   M   H   H   F   S   D   A   A   E   A   Y   I   E   M   R   N   S   E   G     240

 721  TTC TAC ATG CCC AGG TAT GGA CCC CTA AGG AAT TTA AGG GAC AAG AGC TTG GCA AGG TAT     780
 241   F   Y   M   P   R   Y   G   P   L   R   N   L   R   D   K   S   L   A   R   Y     260

 781  GCT TTC GAC TTC TAC GAG GTA ACA TCC AAA ACT TCT GAT AGA GCA AGA GAA GCA ATA GCA     840
 261   A   F   D   F   Y   E   V   T   S   K   T   S   D   R   A   R   E   A   I   A     280

 841  CAA ATG AAG GCC GCA GCT CTC GCC AAC GTA AAC ACC AGG ATG TTT GGC TTG GAT GGG AAC     900
 281   Q   M   K   A   A   A   L   A   N   V   N   T   R   M   F   G   L   D   G   N     300

 901  GTG GCA ACA GTT AGT GAG AAC ACT GAG AGG CAT ACT GCT GCT GAT GTG AAT CAG AAC ATG     960
 301   V   A   T   V   S   E   N   T   E   R   H   T   A   A   D   V   N   Q   N   M     320

 961  CAC TCC CTC TTA GGG ATG ACG CAT GGG CAG TGA AGG CTT GGG TTC CGC CCT CGC CAC AGT    1020
 321   H   S   L   L   G   M   T   H   G   Q   *

1021  TAT CGT CTC ATG ACC TTA GCT AAT GGT TGG TTT TAG TAA ATA GTA CGC ATA TAT CCA GTC    1080

1081  GAA CTA TAT ATG TTA GTG TGG TTT TCC ACC AGT ATG TTA TGT TAC TTT AAT TGA ACT TAG    1140

1141  CGG AGA GGC CAT ACC CCG GTT GGA GTG CTC AGG GTG ACC GTG TCA CGC GTA GCT ACC GAG    1200

1201  ATT TGT GTA TGA AAA CTC TCT                                                       1221
```

Figure 11: 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.

The CP starts at nt 166 (codon 56) and ends at nt 992 (codon 331) . It is therefore 828 nt long, and is expected to translate to a protein 275 amino acids long, with an expected size of 31.2 kDa.

4.4 Cloning and expression of the coat protein gene in *E. coli*.

A DNA fragment of about 800 bp (Figure 12), corresponding to a translatable coat protein (CP_{nd}) gene, was obtained by PCR amplification using the previously cloned 3' terminal 1.2 kb clone of CABMV-Z3 (Section 4.3) as template. The primers used in this amplification incorporated an upstream *Nde*I restriction endonuclease recognition site, which after restriction endonuclease digestion leaves an upstream ATG codon, and a *Bam*HI site downstream.

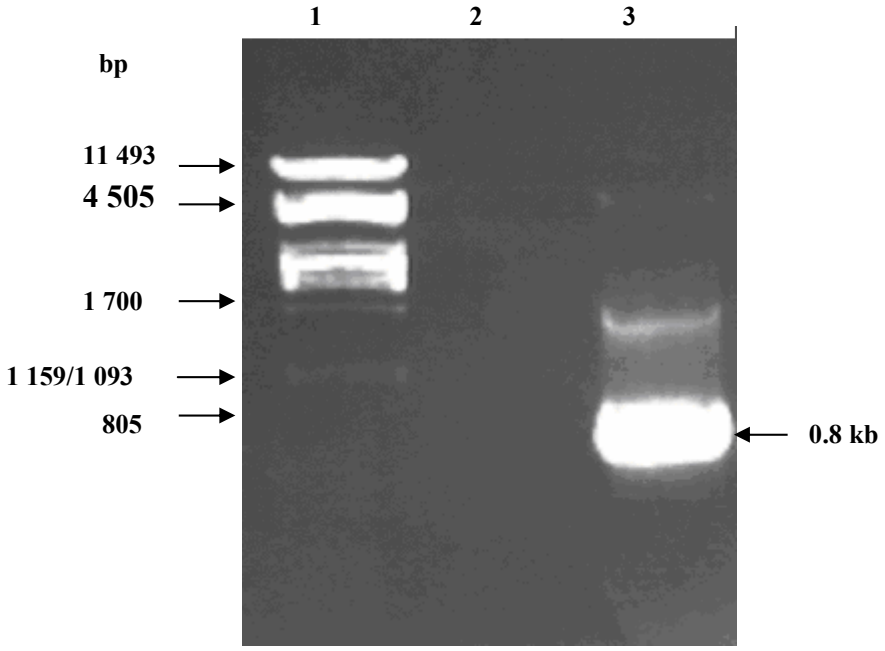

Figure 12: Photograph of a 0.8% (w/v) agarose gel stained with ethidium bromide showing the amplified 800 bp CABMV-Z3 CP gene for expression in bacteria. The lanes are: **1** –molecular weight marker (λ-*Pst*I), **2** - negative control, **3** – CABMV-Z3 amplified from a pGEM-CPRep template.

The amplified CP gene was successfully ligated into pGEM-T, as verified by digestion of DNA from white colonies with *Nde*I which excises out the 800 bp insert (Figure 13).

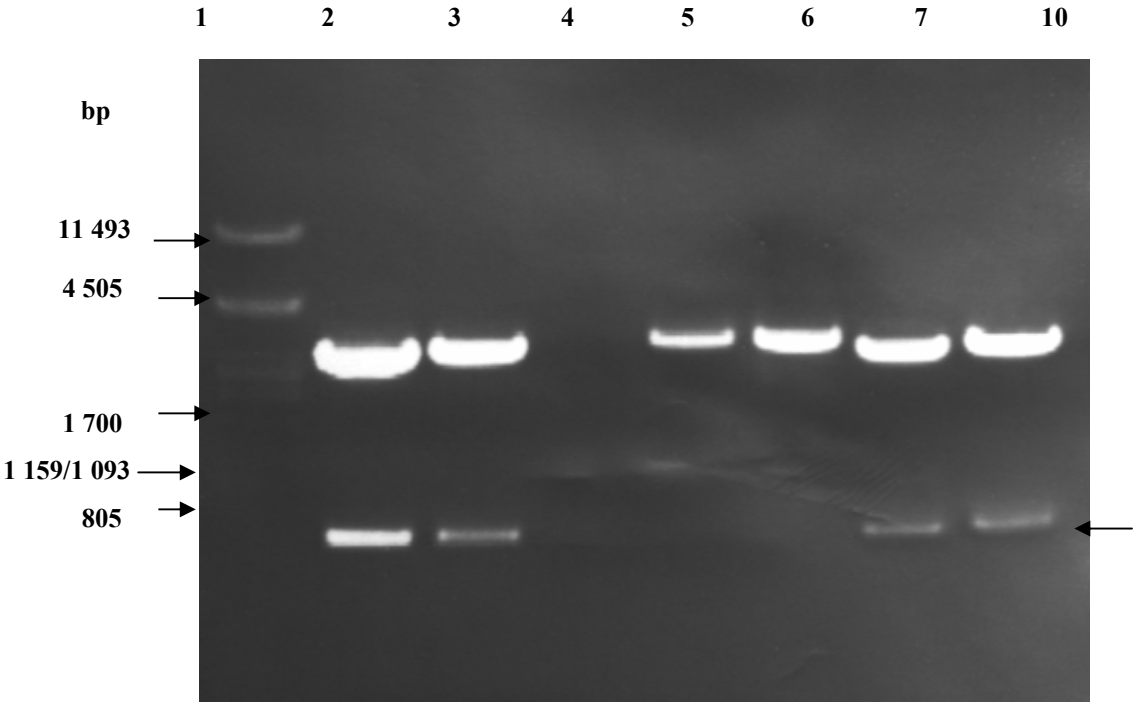

Figure 13: Photograph of a 0.8% (w/v) agarose gel of a plasmid DNA from putatively recombinant pGEM-CP_{nd}, digested with *Nde*I to excise the 800 bp CP_{nd} insert (arrow). The lanes are: **1** – molecular weight marker (λ-*Pst*I), **2** to **8** – plasmid DNA from white colonies. The required recombinants are in lanes 2, 3, 7 and 8.

The next stage of the cloning experiment was to sub-clone the CABMV CP gene into the prokaryotic expression vector pT7.7. In order to do this, the CP fragment of pGEM-CP was excised by *Bam*HI/*Nde*I digestion and purified by recovery from gel together with a similarly digested pT7.7 (Figure 14).

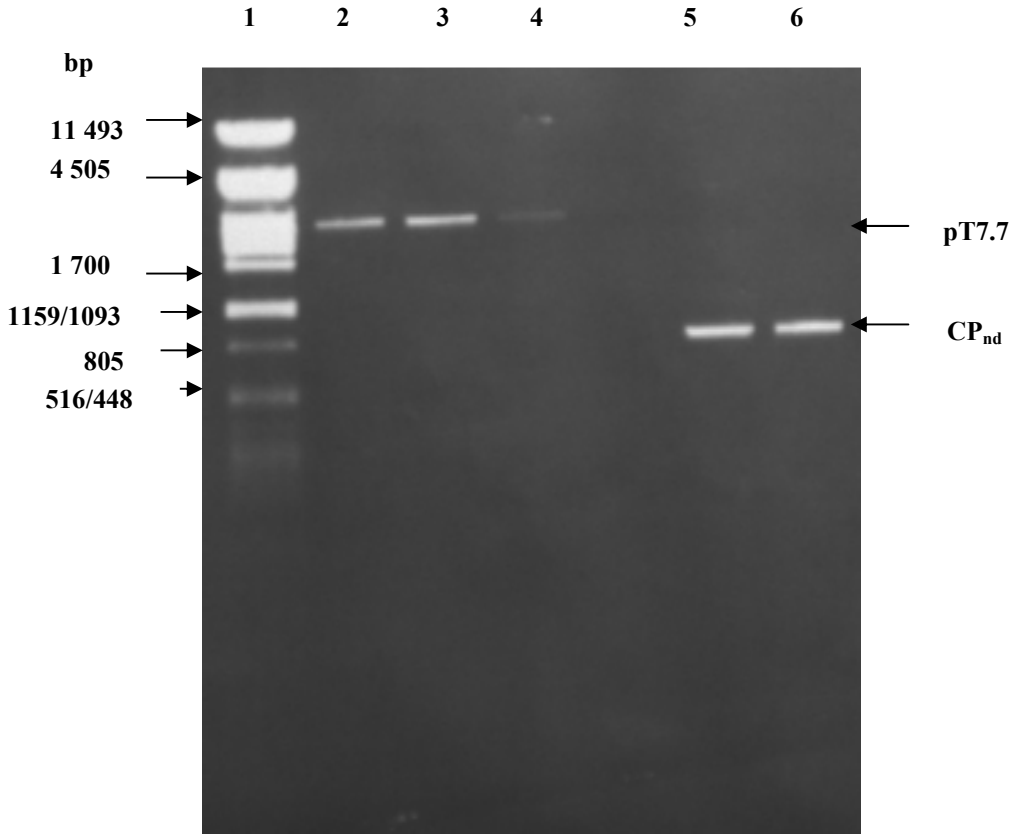

Figure 14: Photograph of a 0.8% (w/v) agarose gel showing *Bam*HI/*Nde*I digested CP_{nd} and pT7.7 recovered from gel. Lane **1** – MWM (λ *Pst*I digest), lanes **2** and **3** - linearised pT7.7, lanes 5 and 6 – CP_{nd} recovered from gel after digestion with *Bam*HI and *Nde*I.

The recovered DNA fragments were successfully ligated and used to transform competent *E. coli* DH5α cells. DNA was isolated from the transformants and recombinant pT7.7-CP_{nd} clones were identified after *Nde*I and *Bam*HI/*Nde*I digestion of the plasmid DNA (Figure 15).

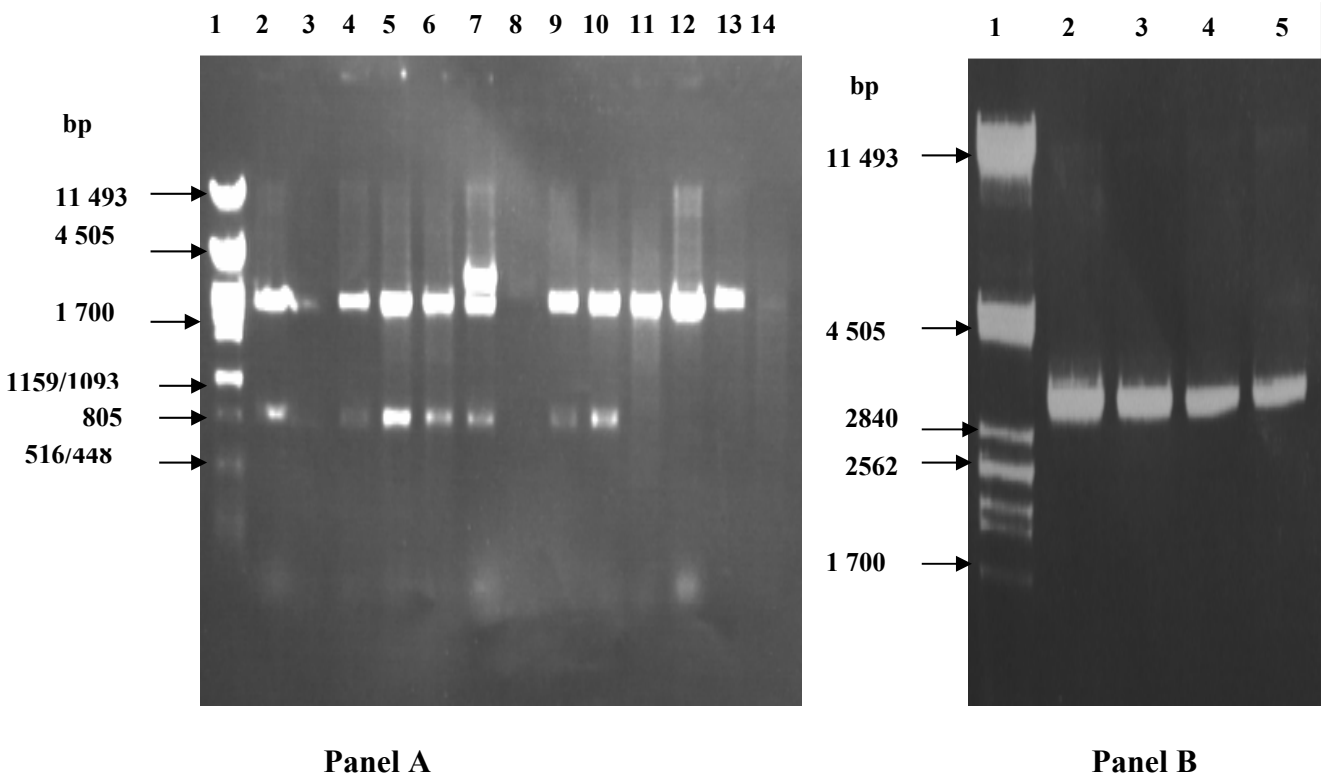

Figure 15: Photograph of a 0.8% (w/v) agarose gel of plasmid DNA isolated from transformants and digested with *Bam*HI/*Nde*I (Panel A) and *Nde*I (Panel B) in an experiment to identify recombinant pT7.7 clones with CP insert. The lanes are, **Panel A: 1** – molecular weight marker (λ-*Pst*I), **2** to **12** – DNA from white colonies; **Panel B: 1** - molecular weight marker (λ-*Pst*I), **2** to **5** - DNA from white colonies with CP inserts digested with *Nde*I.

The recombinant plasmid pT7.7-CP_{nd} was transformed into the expression host *E. coli* BL21. Transformed BL21 lines were identified after CTAB DNA minipreps, and the DNA run on gel after *Bam*HI/*Nde*I digestion (Figure 16).

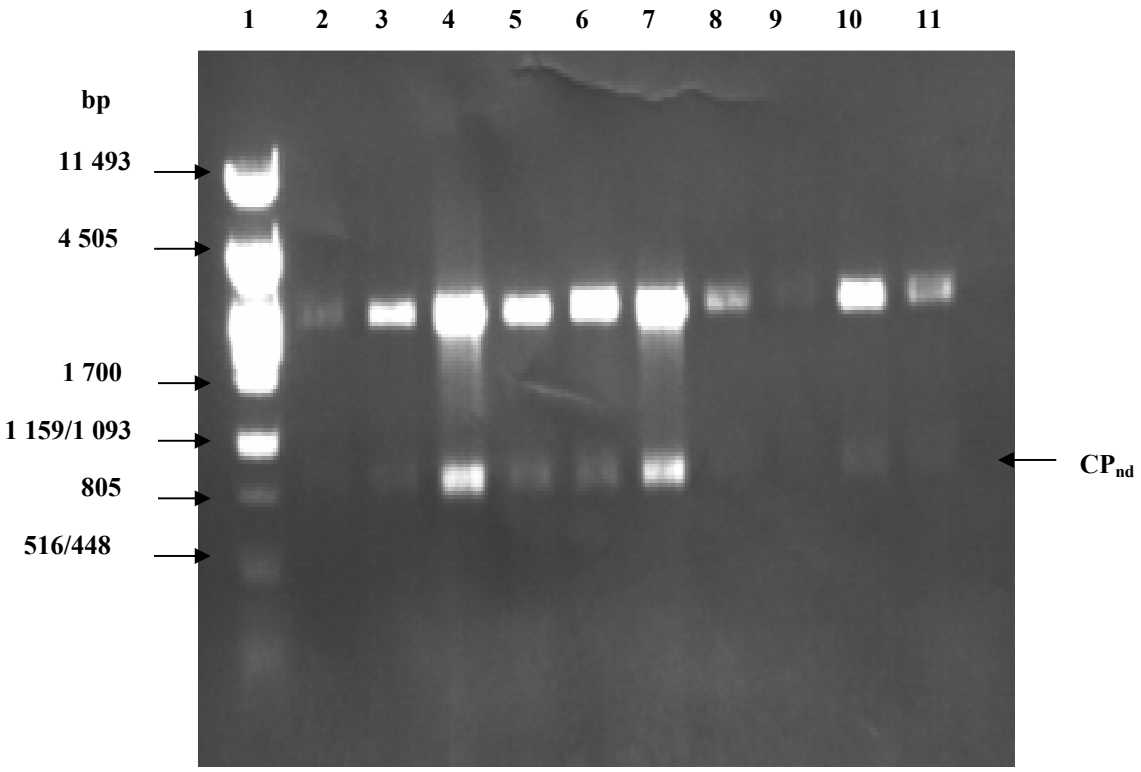

Figure 16: Photograph of a 0.8% (w/v) agarose gel showing pT7.7-CP clones isolated from *E. coli* BL21 cells and cut with *Bam*HI/*Nde*I. The lanes are: **1** – molecular weight marker (λ-*Pst*I), **2** to **11** – DNA form different colonies and digested with *Bam*HI/*Nde*I. The arrow shows the position of the CP_{nd} excised from pT7.7-CP_{nd}.

Clones 4 and 7 were selected for use in expression experiments. SDS-PAGE results showed expression of large quantities of cloned CABMV CP in *E. coli* BL21, and relatively pure coat protein could be isolated from the inclusion bodies fraction of lysed cells and the purified CP was used to raise antiserum in a rabbit.

4.5 PCR cloning of the various forms of CABMV coat protein genes

Amplification of CP_k, CP_{stop} and PC were successful, with products of about 800 bp being amplified (Figure 17A). The amplification of a truncated CP using primers CPcore P1 and CPcore P2 was also successful, giving a product of about 450 bp (Figure 17B).

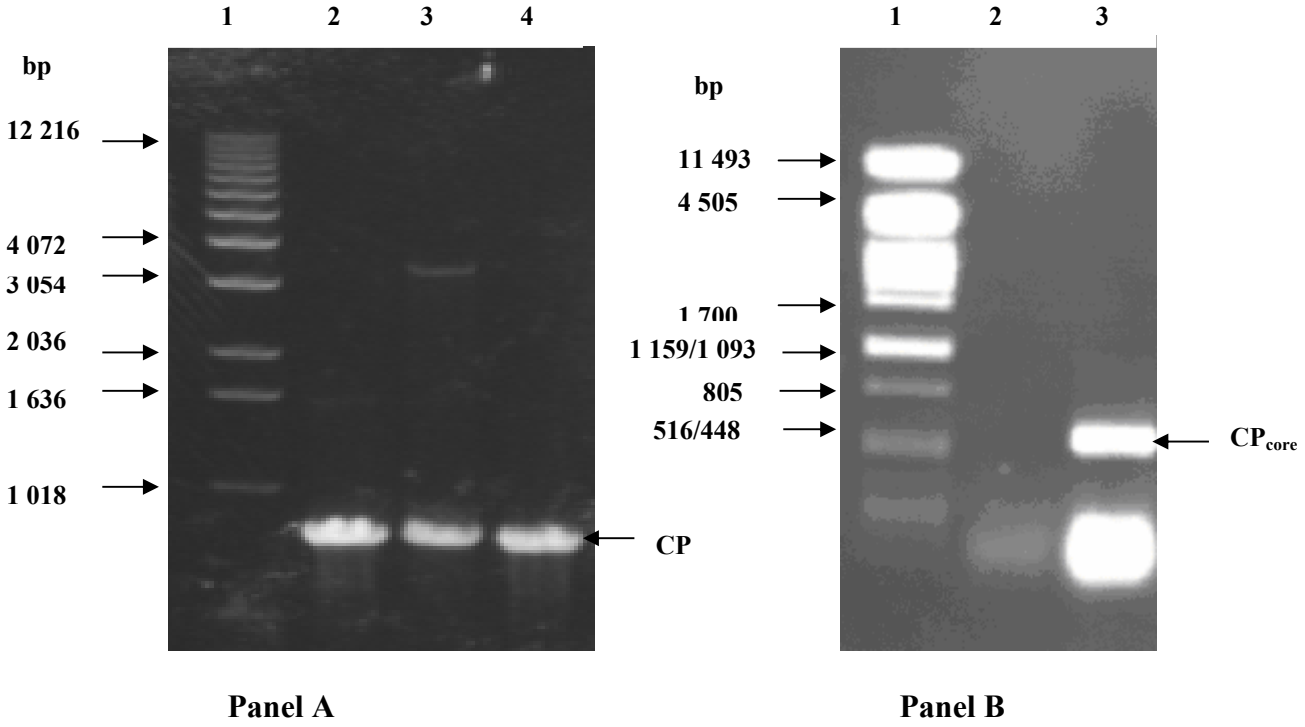

Figure 17: Photograph of 0.8% (w/v) agarose gels of PCR-amplified CABMV CP genes. The lanes are, **Panel A: 1** – MWM (1kb ladder); **2** – CP_k; **3** – CP_{stop}; **4**- PC
Panel B: 1 – molecular weight marker (λ-*Pst*I), **2** – water control, **3** – CP_{core}.

The amplified DNA was digested with *Bam*HI and *Sal*I, and ligated into a similarly digested plasmid pCa2Nos, between the 35S promoter and Nos terminator, to give plasmid constructs that were designated pCa2Nos-CP_k, pCa2Nos-CP_{stop}, pCa2Nos-PC and pCa2Nos-CP_{core}, respectively.

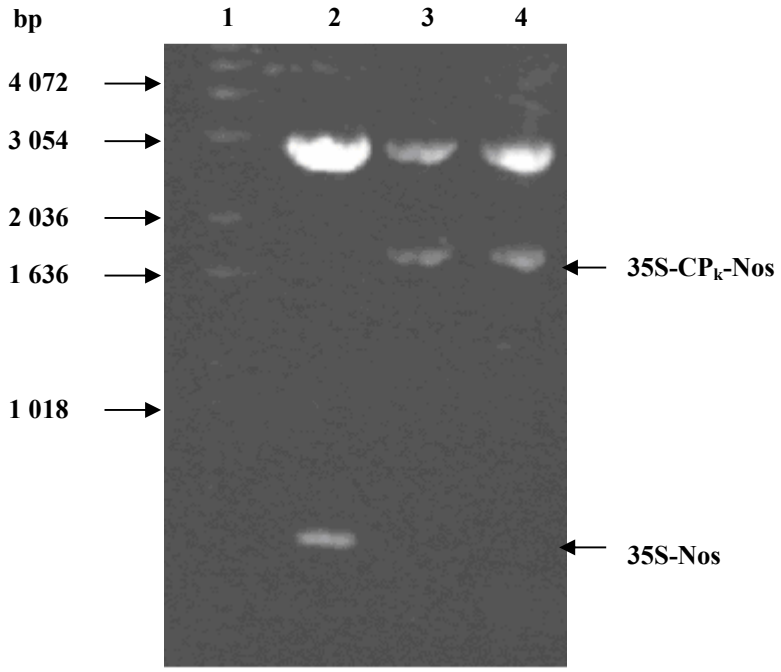

Figure 18: Photograph of a 0.8% (w/v) gel of plasmid DNA isolated from colonies transformed with pCa2Nos-CP_k ligation mix and digested with *Hin*dIII. The lanes are: **1** – molecular weight marker (1 kb ladder); **2** – pCa2Nos; **3** and **4** – DNA from colonies with the expected size fragments for pCa2Nos-CP_k. The arrow shows the 35S-CP_k–Nos fragment excised from pCa2Nos-CP_k.

Figure 18 shows the recombinant pCa2Nos-CP_k plasmid digested with HindIII to release the 35S-CP_k-Nos cassette. Plasmids pCa2Nos-CP_{stop}, pCa2Nos-PC and pCa2Nos-CP_{core} were identified in similar experiments. The recombinant pCa2Nos plasmids were digested with *Hin*dIII to release the 35S-(modified) CP-Nos fragment which was then ligated into the unique *Hin*dIII site of the binary plasmid pBI121. Recombinant binary plasmids isolated from transformed *E. coli* were identified after screening by *Hin*dIII digestion, and further analysed by *Pst*I and *Xba*I restriction endonuclease analysis (Figure 19).

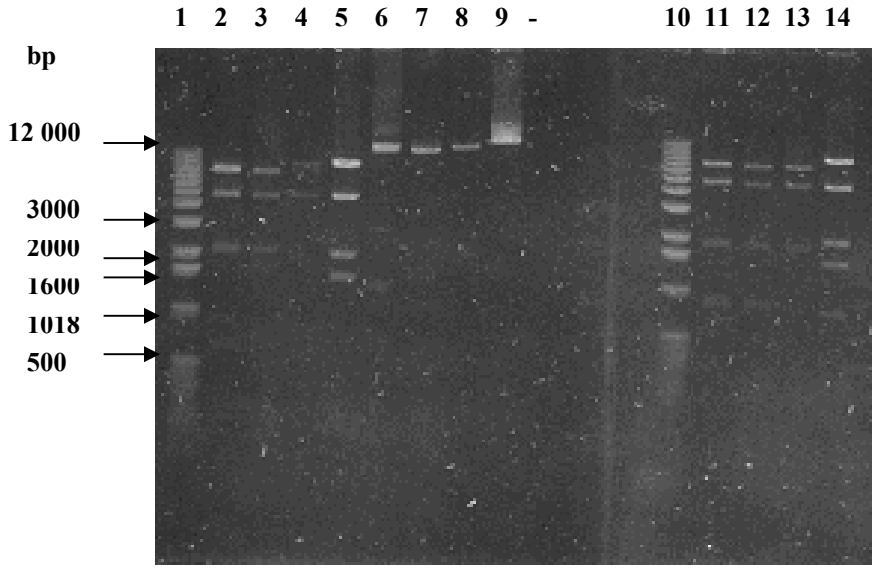

Figure 19: Photograph of 0.8% (w/v) gels of plasmid DNA isolated from *E. coli* cells transformed with the pBI121, pBI121-CP_k, pBI121-PC, pBI121-CPRep digested with *Pst*I, *Xba*I and *Pst*I/*Xba*I. The lanes are: **1&10** – 1 kb molecular weight marker, **2 -** pBI121-PC, **3** – pBI121-CP_k, **4** – pBI121, **5** - pBI121-CPRep, **6 -** pBI121-PC, **7** – pBI121-CP_k, **8** – pBI121, **9** - pBI121-CPRep, **11 -** pBI121-PC, **12** – pBI121-CP_k, **13** – pBI121, **14** - pBI121-CPRep. The DNA in lanes 2 - 5 was digested with *Pst*I, 6 – 9 *Xba*I and 11 - 14 *Pst*I/*Xba*I double digest.

Figure 19 shows the results of restriction digests of pBI121-CPk and pBI121-PC compared to pBI121 vector. Similar analyses were done with pBI121-CP_{stop} and pBI121-CP_{core}, and the fragments, compared with plasmid vector pBI121 were as expected. Figure 20 overleaf shows the plasmid maps of the binary constructs made in these experiments.

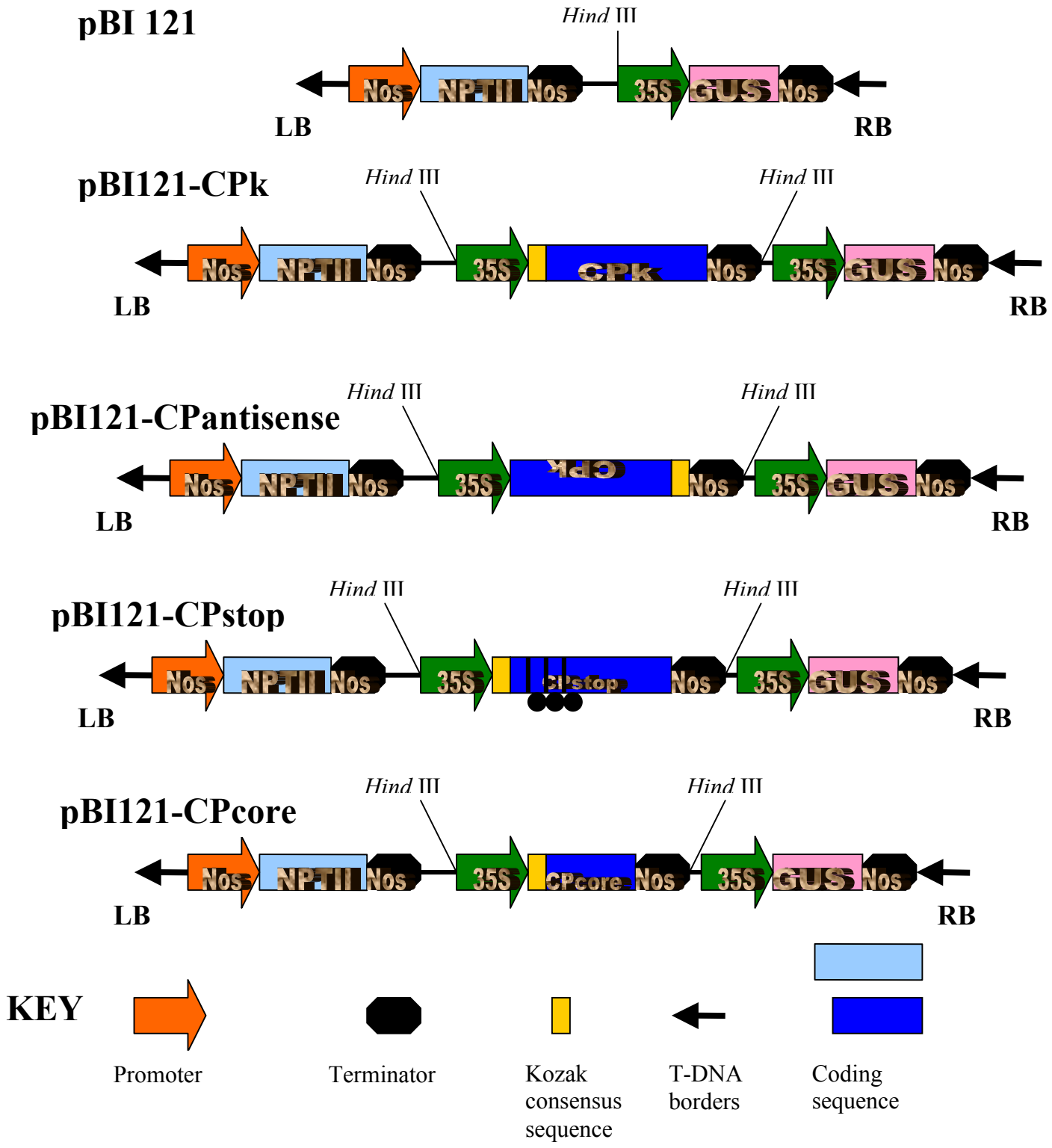

Figure 20: Plasmid maps of binary constructs used for plant transformation.

4.6 Sequencing of CABMV pBI121-CP constructs

CABMV CP-recombinant binary plasmid DNA was isolated from *E. coli* DH5α strains using the Wizard™ Plus DNA Purification System (Promega, Wiscounsin, 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 corresponding CP sequence was obtained for pBI121-CP_k, pBI121-CP_{stop} and pBI121-PC, and verified to be correct after comparison to the sequence in Figure 11 and as published by Sithole-Niang *et al*. (1996). The sequence of pBI121-CP_{core} did not match that of the core region of CABMV-Z3, and was probably a cloning artifact. The construct was therefore not considered in subsequent experiments.

Another set of sequencing primers were designed and used for sequencing across the CP-35S promoter and CP-Nos terminator junctions, and verification of promoter and terminator sequences.

4.7 Cloning of full length CABMV genome

The long range RT-PCR to amplify the CABMV genome resulted in an amplification product of 10 kb (Figure 21). However smaller sized products of 2 kb, 3 kb were also amplified at higher magnesium chloride concentrations.

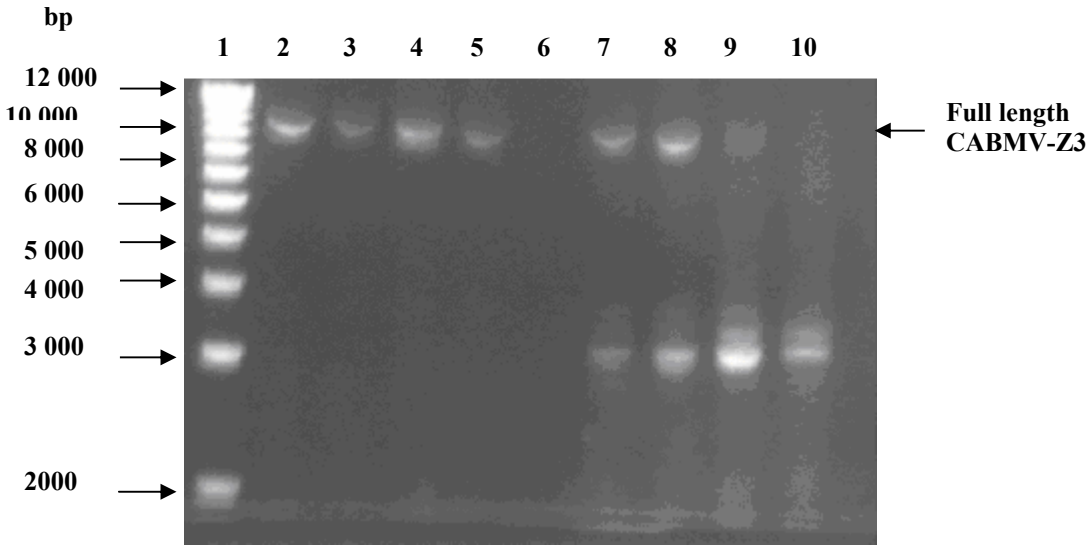

Figure 21: Photograph of a 0.8% (w/v) gel showing the results of long range RT-PCR to amplify the entire CABMV genome. The lanes are: **1** – 1 kb molecular weight ladder, lanes **2** to **10** – amplifications under varying magnesium chloride concentrations: 0, 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 2.5 and 5.0 mM $MgCl_2$. The arrow shows the position of the 10kb amplification product.

The 10 kb product was recovered from gel, and ligated in pEV1, and used for transfection. DNA was isolated from putative recombinants and digested with *Not*I and run on gel (Figure 22).

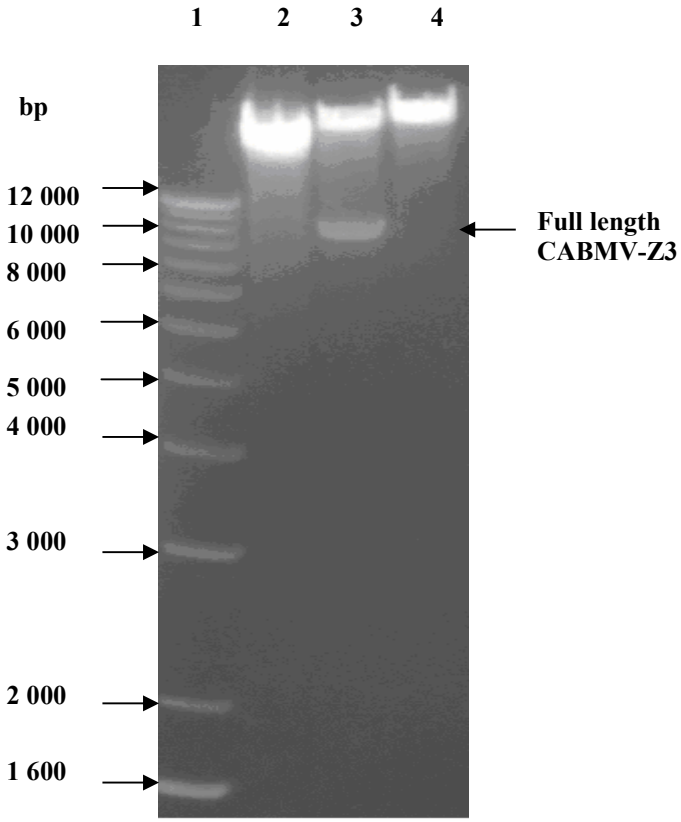

Figure 22: Photograph of a 0.6% (w/v) agarose gel of putative recombinant pEV1 with a 10 kb insert consisting of the entire CABMV-Z3 genome, digested with *Not*I. The lanes are: Lane **1** – 1 kb ladder, lanes **2** to **4** – different clones. The required clone is shown in lane 3, where an insert of 10 kb was released from the vector. The arrow on the right indicates the position of the 10 kb full-length insert.

The vector pEV1 in which the 10 kb amplicon was cloned was named pEV1-Z3 and was further characterised by restriction endonuclease analysis. In the sub-cloning experiment, the 10 kb insert of pEV1-Z3 was digested out by *Not*I digestion and recovered from gel together with *Not*I linearised pSPT18 (Figure 23).

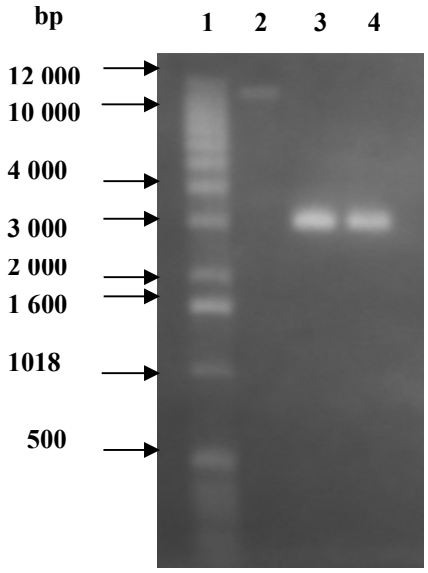

Figure 23: A photograph of a 0.6% (w/v) agarose gel showing the 10 kb insert of pEV1-Z3 and linearised pSPT18 and 19 recovered from gel. The lanes are: **1** – molecular weight marker (1 kb ladder), **2** – 10 kb insert released from pEV1-Z3, **3** – linearised pSPT18, **4** – linearised pSPT19.

After ligation and transformation of the DNA fragments shown in Figure 23, the putative recombinants were screened by restriction endonuclease digestion. Two good clones, 11-1 and 51-1 were selected and further analysed by *Eco*RI and *Hin*d III restriction endonuclease analysis (Figure 24), and sequencing across the junctions.

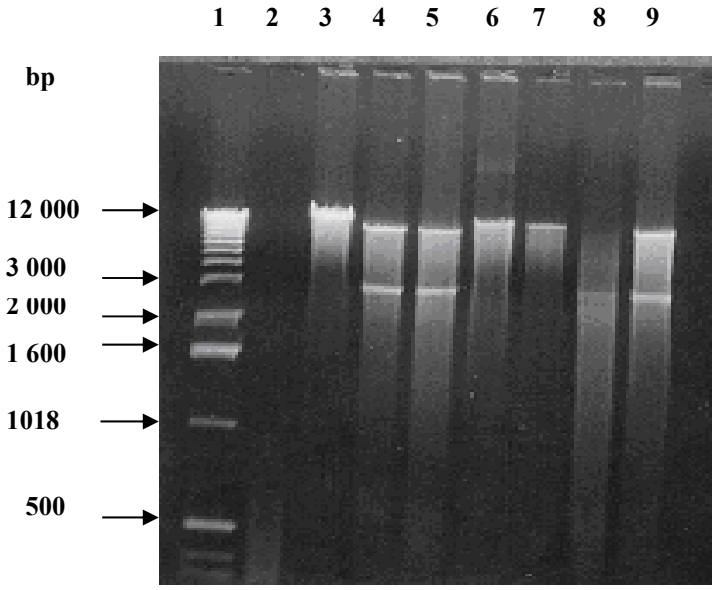

Figure 24: Photograph of a 0.6% (w/v) agarose gel showing pSPT18-Z3 clones digested with various restriction endonucleases. The lanes are: **1** – 1 kb ladder molecular weight marker, **2** – *Eco*RI, **3** – *Hin*dIII, **4** – *Hin*dIII/*Eco*RI, **5** – *Hin*dIII/*Eco*RI, **6** – *Eco*RI, **7** – *Hin*dIII, **8** – *Hin*dIII/*Eco*RI, **9** – *Hin*dIII/*Eco*RI. DNA of clone 11-1 was loaded into lanes 2 to 5, while that of clone 51-1 was loaded into lanes 6 to 9. The DNA was however degraded, probably due to contamination of the *Eco*RI enzyme, and extended incubation.

This clone was also analysed by sequencing across the cloning junctions, and is available for use in experiments to produce full-length transcripts of the clone.

4.8 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 25). This verification was necessary since deletions can occur in binary vectors. The verified clones were then used in plant transformation experiments.

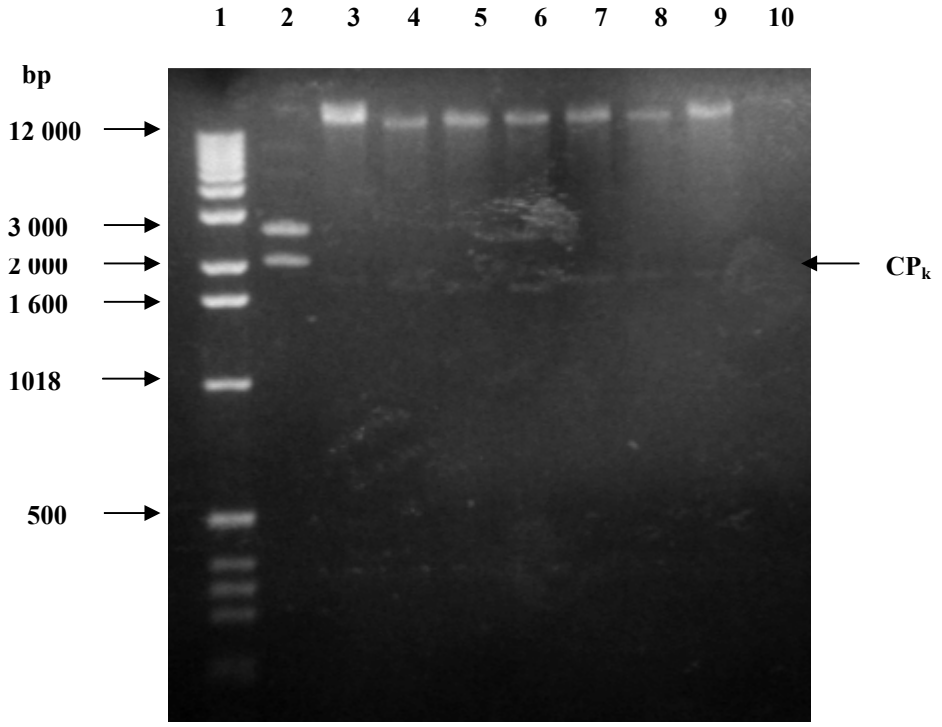

Figure 25: Photograph of a 0.8% (w/v) agarose gel of *Hind*III-digested plasmid pBI121-CP_k DNA isolated from *E. coli* DH5α transformed with DNA from *A. tumefaciens* LBA 4404. The lanes are: **1** – molecular weight markers, **2** – pCa2Nos-CP, **3** to **10** – DNA from different *E. coli* colonies transformed with pBI121-CP_k and then used to transform *E. coli* in order to get enough quantities for this analysis. The arrow shows the position of the 35S-CP_k-Nos fragment excised by *Hin*dIII digestion of pBI121-CP_k.

Similar analyses were done for pBI121-CP_{stop}, pBI121-PC and pBI121 before use in plant transformation experiments.

4.9 Analysis of transgenic *N. benthamiana* plants

4.9.1 Kanamycin resistance assay, CP immunoblot and Northern analysis

A total of 152 *N. benthamiana* R0 lines were produced, whose R1 were tested, and 63 of these were shown to be putatively transgenic since they had kanamycin resistant progeny (Table 8). 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 deduction should be confirmed by Southern hybridization experiments. Lines 028, 034, 035, 049 appear to be multiple-copy lines, based on kanamycin resistance ratios of progeny.

Table 8: Kanamycin resistance and CP immunodotblot analysis results of R1 plants.

Construct	Number of lines	Kanamycin resistant lines	CP immunodotblot +ve lines
pBI121-CP$_k$	(015–082)	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, 080, 079	017, 031, 035, 040, 052, 053, 055, 057, 058, 061, 064, 065, 066, 067
	68 lines	42 lines	14 lines
pBI121- CP$_{stop}$	(210-235)	211, 214, 217, 218, 219, 224, 266, 227, 228, 230, 231, 232, 234	
	26 lines	13 lines	0 lines
pBI121- PC	(411-459)	411, 412, 420, 425	
	49 lines	4 lines	0 lines
pBI121	(810-818)	817, 818,	
	9 lines	2 lines	0 lines

4.9.2 PCR for transgene detection

The presence of transgenes was confirmed by PCR screening for the presence of CP gene. A PCR to detect the CP core region was optimized and used to screen all seedlings before virus challenge experiments. Figure 26 shows a representative result of the procedure.

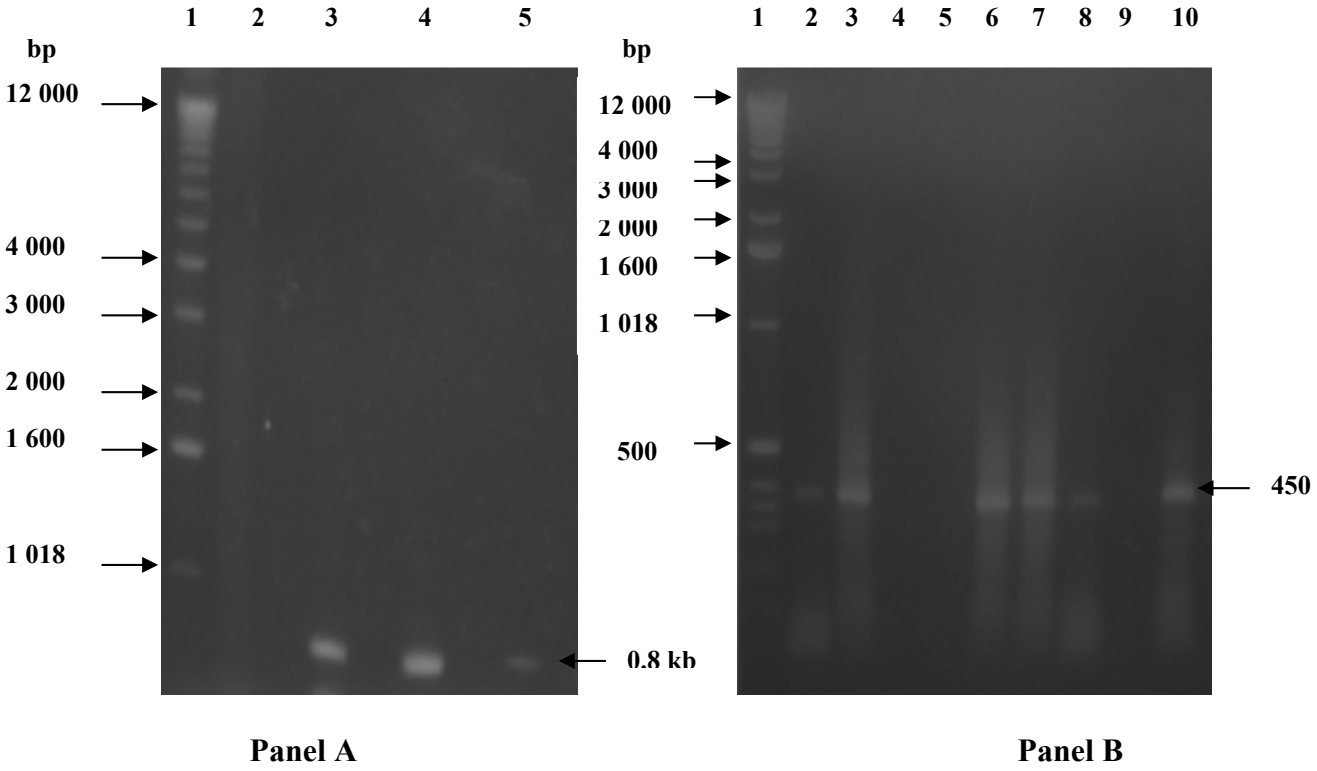

Figure 26: Photograph of 1.0% (w/v) agarose gels of PCR products to detect the presence of transgenes in putatively transformed *N. benthamiana* plants. **Panel A:** PCR to detect for the presence of the entire CABMV CP gene. The lanes are: **1** – molecular weight marker (1 kb ladder), **2** – negative 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, 8 and 10.

The PCR products were blotted onto nitrocellulose membrane and probed with the DIG-labelled CP_{core} probe to confirm the identity of the amplicon (Figure 27).

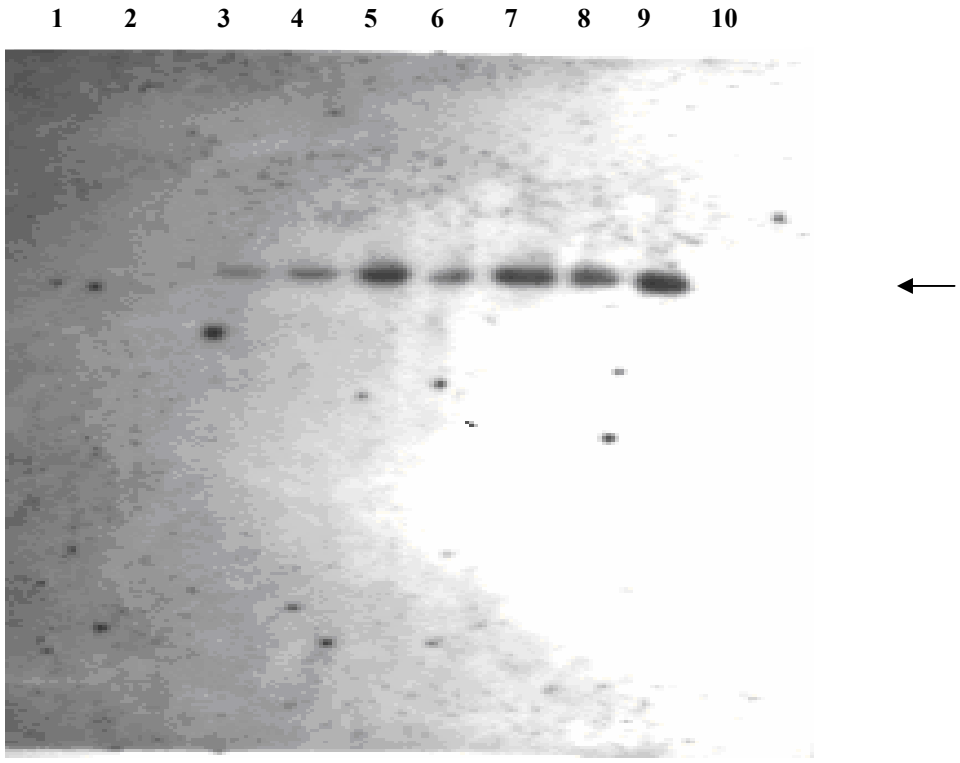

Figure 27: X-ray radiogram of a blot of the products of a PCR to amplify the CPcore region of putatively transformed *N. benthamiana* plants. The lanes are**: 1** – 1kb ladder, **2** – non-transformed *N. benthamiana*, **3** – line 034, **4** – line 040, **5** – line 043, **6** – line 046, **7** – line 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. The DIG-labelled CP_{core} fragement was used as probe.

4.9.3 Southern blotting for copy number determination

Southern hybridization using DIG chemiluminescent procedure failed to detect transgenes. This is probably due to low sensitivity of the procedure. Use of a more sensitive detection procedure such as the ^{32}P procedure might be able to detect the signal.

4.10 Virus challenge experiments

Between 7 and 42 individual R1 plants were used per challenge experiment. Some sap-inoculated plants showed systemic symptoms at two weeks, not within the 5 – 7 days of challenge observed for control non-transgenic plants. One pBI121-CP_k transformant of note is Line 035 that in addition to delayed symptom development, had symptoms which 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.

Table 9: 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, Tol		Some 035?		Delayed symptom development. Symptoms obvious on all plants at Day 28. No immunity.
	23 lines	Nil	Nil	Nil	
PBI121-CP_{stop}	211-6, 211-7, 226-3,	211-3, 211-3, 211-4, 226-8	227-3, 227-4, 227-5		Delayed symptom development, some tolerance. Progeny of 227 display recovery.
	3 lines	4 lines	3 lines	Nil	
PBI121- PC	425-1			411-4, 420-1, 412-3	Not much resistance. Lines with modified symptoms were noted.
	1 line	Nil	Nil	3 lines	
PBI121	Nil	Nil	Nil	Nil	No resistance.

The other observations made include the following. Plant 028-17 had developed a new stunted shoot by day 28, indicating incomplete recovery. Only progeny of plant 049 was challenged with sap, virions and vRNA. The virions were still infective, as evidenced by individual 049-2 which developed symptoms after challenge with vRNA. Lines transformed with pBI 121 are controls to demonstrate that resistance observed is not due to the presence of the binary vector.

4.11 Cowpea transformation

More than 1 200 cowpea seedlings were subjected to transformation by electrophoresis. The cowpea plants that gave a GUS positive reaction and the treatments to which these plants had been subjected are summarised in Table 10. However, the GUS positive reactions were only in sectors of the leaf. Therefore some sectors may have been missed at sampling by punching. PCR screening, with CP and CP_{core} primers, showed positive amplifications in these plants. However, only one plant gave a positive signal in Southern analysis.

Table 10: Cowpea plants that have tested GUS positive

Plant No.	Variety/ Date planted	Transformation date/ Original Plant number	DNA construct	Current/ Time/ Distance between electrodes	Size	Stem/ 1st true leaves/ Cotyledons	Notes
217	475/89 8/6/01	8/13/01 4	CP_{core}-pBI121, Circular	0.15 V 15 min 7 cm	8 cm	Straight Open On	No pre-treatment. Harvested 11/06/01
309	475/89 8/10/01	8/13/01 A79	CP_k-pBI121, *Nhe*I linearized	0.15 V 15 min 7 cm	6 cm	Straight Open On	No pre-treatment. Harvested11/15/01 and 12/15/01
301	475/89 8/13/01	8/21/01 A114	CP_k-pBI121, *Nhe*I linearized	0.15 V 15 min 7.5 cm	5 cm	Straight Open On	No pre-treatment. AC 30 sec
398	475/89 8/18/01	8/26/01 B97	CP_k-pBI121, *Nhe*1 linearized	0.15 V 15 min 6 cm	9 cm	Straight Open On	Punched meristem

A common feature of the GUS positve plants in Table 10 is that the manipulations were carried out on plants that had straight stems, first true leaves open and cotyledons still attached to the seedling. No pre-treatment other than maybe punching the meristem appear to be necessary.

The other lines that also tested GUS-positive, although with very weak reactions are 166 and 174; Line 213 tested negative for GUS. However, because the GUS reaction was slow, with new positives still being identified at the time the experiment was terminated, the numbers obtained thus far are not truly indicative of the efficiency of this procedure.

The GUS gene fragment could be amplified from lines 166, 174, 213, 217, 301 and 309, and GUS+ve potato, but not from a water control and GUS-ve potato (Figure 28). The CABMV

CPcore gene fragment could be amplified from lines 166, 174, 217, 301 and 398 (Figure 29).

Southern analysis confirmed the presence of CP gene in line 398 (Results not shown).

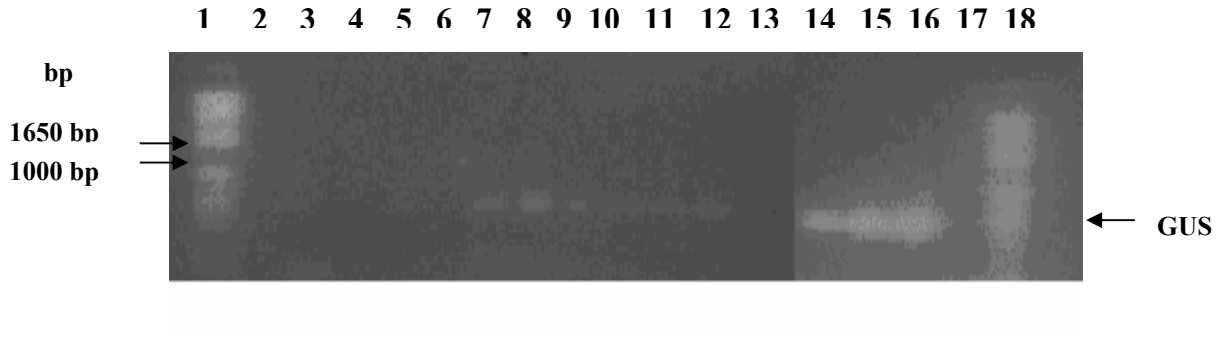

Figure 28: Photograph of a 0.8% (w/v) agarose gel of PCR products to screen for GUS gene fragment in GUS+ve transgenic cowpea plants. The lanes are: **1** – molecular weight marker (Kb+), **2** - 213y, **3** – water control, **4** - 398c, **5** - 398b, **6** - 398a, **7** - 174, **8** - 166, **9** - 217, **10** - 309, **11** - 301, **12** - 213, **13** - Pot-, **14** – **16** titrations of GUS+ve potato, **17** – water control, **18** – Kb+ molecular weight marker. The arrow indicates the position of the amplified GUS gene fragment.

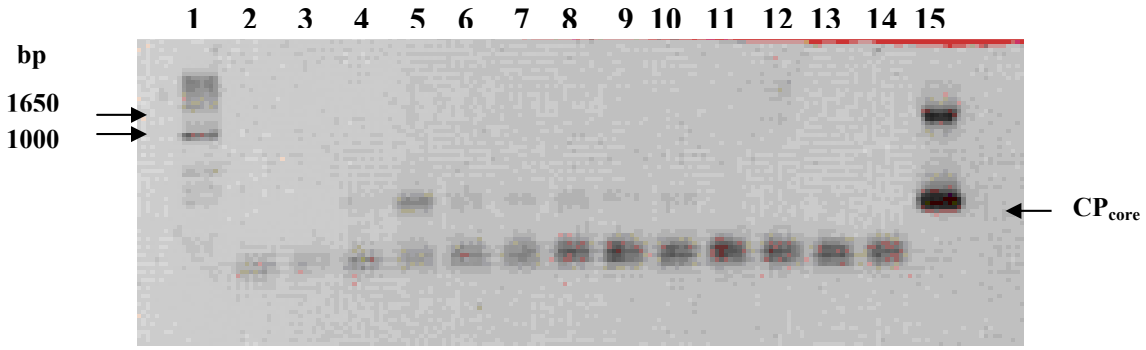

Figure 29: Photograph of a 0.8% (w/v) agarose gel of PCR products to screen for CABMV CP transgenic cowpea plants. The lanes are: **1** – molecular weight marker (Kb+), **2** – water control, **3** – GUS-ve potato, **4** – 213, **5** – 166, **6** – 174, **7** – 217, **8** – 301, **9** – 309, **10** – 398a, **11** – 398b, **12** – 398c, **13** – water control, **14** – GUS +ve potato, **15** – pBI121-CP_k +ve control.

The pre-treatments except punching the meristem do not seem to enable transformation efficiency. Both DC and AC are effective in delivery DNA to the plant cells.

5 DISCUSSION

5.1 Host range studies

Vigna unguiculata, *N. benthamiana* and *Ch. quinoa*, were shown to be possible systemic hosts of CABMV. This is consistent with the observations of Bock & Conti (1974) and Lovisolo (1976) who also described the symptoms of CABMV infection on *V. unguiculata*. The symptoms of the Zimbabwean isolate of the virus, CABMV-Z3, were described for the first time in this thesis. *Nicotiana clevelandii* was also shown to be a host that however showed mild symptoms even at high viral titre. *Chenopodium amaranticolor* was shown to be a good local lesion host for CABMV-Z3. Good local lesion hosts do not result in general necrosis after inoculation, but develop lesions at inoculated sites, and the size and number of the lesions is proportional to quantity of inoculum (Hull, 2001; Agrios, 1997).

Of the hosts of CABMV determined above, *V. unguiculata* and *N. clevelandii* are of economic importance. *Vigna unguiculata* is grown mainly by subsistance farmers. The common practice of growing cowpea interspersed with a non-host crop like maize (Jackai *et al.*, 1985) in a way helps to reduce the spread of viruses. However, if cowpea production is commercialised and the crop is grown in monoculture, disease control becomes more important (Dube 1995).

The symptoms observed on *N. clevelandii* are not severe, but the results of virus isolation experiments reveal very high virus titre in infected plants. This crop may therefore serve as a reservoir of the virus, with the potential to result in sudden severe outbreaks in more susceptible crops such as cowpea if grown in adjacent fields. However, the natural incidence of CABMV infection in *N. clevelandii* was not investigated.

Nicotiana glutinosa, *N. tabacum* cv White Burley, *N. rustica*, *Phaseolus vulgaris* and *C. sativus* were shown to be non-hosts of CABMV. These are the crops that should be utilized in seasons following severe CABMV outbreaks. Other workers have also observed that CABMV does not infect *N. glutinosa*, and in fact CABMV can be distinguished from other cowpea viruses by the fact that CABMV does not infect *N. glutinosa* (Bock & Conti, 1974 and Lovisolo, 1976).

5.2 Virus purification and polyclonal antibody production

Virus concentration was estimated based on the extinction coefficient of 2.4 (mg/ml, 1 cm light path) at 260 nm (Bashir & Hampton, 1995). The highest yields of 25.1 mg/kg of infected leaf material were obtained from *N. clevelandii* even though this host showed very limited symptoms. These yields are higher than the 2 – 17 mg of sweet potato feathery mottle virus per kg of infected *N. benthamiana* leaves obtained by Chavi and co-workers (1997) using similar methodology. This suggests that the mild symptoms are not due to low virus concentrations, but because the virus infection does not induce significant symptoms in this host, even at high virus titre.

Purified potyvirus is expected to have an A_{260} : A_{280} ratio of 1.2 (Lima *et al*., 1979). Highly purified virus was obtained, with A_{260}:A_{280} ratios approximating 1.2. No impurities were visible under the electron microscope even though highly concentrated virus was visible.

Both sodium hydrogen phosphate and potassium hydrogen phosphate buffers work well in procedures to purify CABMV. Sodium hydrogen phosphate buffers are recommended for virus purification from *N. benthamiana*, while potassium hydrogen phosphate buffers give higher yields from *V. unguiculata*. However, the intended use of the purified virus should also be considered, since some applications, for example RNA isolation by SDS methods, are sensitive to

the presence of potassium ions (Sambrook *et al*., 1989). In this study, we recommend the addition of 1 mM EDTA and β–mercaptoethanol to resuspension buffers to prolong storage (results not shown). This is also consistent with the results of Chavi *et al.* (1997) who also showed extensive degradation of stored SPFMV in the absence of EDTA and β–mercaptoethanol.

Use of a 10 – 50% sucrose gradient is an alternative method for virus purification, but the virus obtained following this method was very dilute, and most of the virus was lost during concentration. Virus precipitation using PEG, which is one of the methods for concentrating the virus, resulted in clumping and subsequent loss of virus.

Electron microscopy of PTA stained purified virus particles revealed filamentous particles with dimensions that approximate literature values for CABMV (Bock and Conti, 1974; Losivolo, 1976). The purified virus was used to raise polyclonal antiserum in a rabbit. Both first and second bleeds of the antiserum were of high titre as revealed by the fact that it could be used at a dilution of 10^{-6}, and could detect virus as dilute as 46 ng/ml in an immunodot-blot assay. The size of CP was determined to be 32 kDa in a semi-dry blot. This is in agreement with the calculated size of the CP, based on the nucleotide sequence, and on predictions of Sithole-Niang and co-workers (1996).

Virus purification procedures inevitably result in some fragmentation and degradation of virions in storage. The PEG precipitation results in clumping and bands larger than purified CP may appear, but these could be removed by CsCl density centrifugation. Because sap from an uninfected plant shows a clean reaction, all smearing observed on gel could have been due to fragmentation and clumping of virus fragments.

The polyclonal antiserum produced in this study is an important reagent that can be used in speedy identification of causative agents of disease in future disease outbreaks. It can also be used in studies to compare isolates, and as the basis for the development of a commercial ELISA kit. What would be even more useful in serology is the development of monoclonal antibodies for the detection of particulr isolates.

5.3 Cloning of full length CABMV genome

Cloning of full length CABMV genome was successfully accomplished in pEVI. To ensure efficient expression, the genome was subcloned into pSPT18. More extensive verification and evaluation of pEV1-Z3 and pSPT18-Z3 clones were not done, but restriction endonuclease analysis and sequencing across cloning junctions indicate that these clones include the entire CABMV-Z3 genome. The clones are available for use in experiments to make infectious transcripts and may be evaluated for infectivity. The clones also serve as a stable storage form of the virus isolate. Alternative forms of storage, such as passaging to plants in the greenhouse are laborious and expensive, and the isolate may change due to mutation or may become contaminated. Cross contamination may also occur. Freeze-dried samples retain infectivity longer than fresh samples, but they also tend to lose infectivity over time (Bock and Conti, 1974).

The full-length clone in the form of pEV1-CABMV is also important because it can be used in virus evolution studies. Infectious potyvirus transcripts obtained from a T7 promoter were found to have a high rate of mutation (Schneider and Roossinck, 2001). The use of a clone driven by a CaMV 35S promoter however resulted in infections with a single molecular species (Tan *et al*., 2005). This would be very usefull in evolutionary studies.

5.4 Cloning and sequencing of 3'-terminal region of CABMV

Of the 15 field isolates of CABMV collected by Sithole-Niang and co-workers, 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 and co-workers (1996). The sequence presented in Figure 11 includes the last 55 amino acids of the replicase gene, the whole CP, and the 5'UTR. This experiment was necessary to confirm that the identity of CABMV-Z3 and that the virus had not undergone any significant changes due to mutation during passaging experiments.

Nucleotide sequence comparison in the CP region showed 100% identity to that published by Sithole-Niang *et al*., (1996). The source of variation from isolate to isolate will become apparent when more sequence information of the different isolates become available.

5.5 Cloning and expression of the CP gene in *E. coli*

The cloned 3'-terminal region of CABMV-Z3 was used as template to specifically amplify the CP gene, using primers that incorporated an ATG translation start. Incoporation of an ATG was necessary because in CABMV the CP is expressed as part of a polyprotein that is then post-translationally cleaved by NIa (Urcuqui-Inchima, 2001). However, because CP is the most C-terminal protein of the polyprotein, its coding sequence already includes a stop codon (TGA) and so it was not necessary to add another one. The restriction endonuclease *Nde*I was the enzyme of choice because its recognition site already includes an in-frame ATG.

The objectives of these experiments were to produce CABMV CP in a prokaryotic cell and use it to raise antiserum. The clone CP would provide a ready source of large quantities of the CP for use in virological studies. The recombinant CP would serve as a good antigen since it would be free of plant material which would otherwise result in cross reaction with field material. This antiserum was then compared to the antiserum produced against *in vitro* assembled virus. Results of this comparison show that the two antisera display the same sensitivity to CABMV (results not shown), showing that the CP expressed in the prokaryotic system folds in a similar manner to that of the intact virion.

5.6 PCR Cloning of the various forms of CABMV CP genes

Four forms of the CABMV CP genes were amplified using the cloned 3'-terminal region of CABMV-Z3 as template and cloned between the promoter and terminator of vector plasmid pCa2Nos in binary vector plasmid pBI121. The first 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, can be transcribed and translated into the wild type CABMV CP. This construct would test the CP-MR mechanism. The second construct was like the first, except that it has stop codons in all three reading frames, introduced via the upstream primer. This CP gene can be transcribed but no corresponding protein product is produced since translation is aborted as soon as it is initiated. This construct would test sense RNA mediated resistance mechanism. The third construct is the antisense CP, like the pBI121-CP_k but cloned in the reverse orientation, and thus would test antisense RNA mecdiated resistance mechanism. The integrity of the clones 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 complete CP sequence was obtained for pBI121-CP_k, pBI121-CP_{stop} and pBI121-PC, and verified to be correct

after comparison to the sequence published by Sithole-Niang and co-workers (1996). A fourth construct, pBI121-CP_{core} was designed to code for the core region of the CP. However, this clone could not be verified by sequencing as it appeared to include sequences that were not of CABMV origin. These sequences may have originated from errors of the process of PCR. This is not an uncommon occurrence, and demonstrates the importance of clone verification by sequencing.

5.7 Pathogen derived resistance to CABMV

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 nucleotide sequence of pBI121 is not available, and deletions and other rearrangements can occur in binary vectors (Frisch *et al*., 1995). The good clones were then used in plant transformation experiments.

The number of pBI121-CP_k transformed plants was high (42 out of 68) while those of pBI121-CP_{stop} and pBI121-PC transformants were lower (13 out of 26 and 4 out of 49 respectively). Resistance to kanamycin, detection of the CP in an immunodotblot assay and PCR amplification of 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 were 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 inoculum. These results are consistent with those of Powell-Abel and others (1986) who showed protection in lines

expressing detectable levels of CP. This is also consistent with observations on susceptible lines such as 034 which, 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. 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. Delayed symptom development also exerts little evolutionary pressure 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.

The presence of detectable CP levels however does not necessarily lead to resistance. Progeny of lines 017, 031, 040 in which the CP could be detected in immunodotblot assays, examplifies this. This suggests that there is a more complex interaction that must take place to result in resistance, and these failed to take place in these counter examples. It is likely that such counter-examples also also obtained in other studies, but fail to be reported (Nap et al., 1993). Until such events are also fully analysed, universal mechanisms of PDR cannot be fully elucidated.

The plants transformed with pBI121-CP_{stop}, pBI121-PC and pBI121 did not have any detectable CABMV CP as expected since the constructs do not code for a functional CP. The pBI121-CP_{stop} contruct resulted in plants showing delayed symptom development and tolerance. This is a higher level of resistance and allows the plant to yield viable seed. It is postulated that the

mechanism would be RNA-mediated since this construct cannot be translated to give CABMV CP, as was shown in TEV (Lindbo & Dougherty, 1992; Goodwin *et al*., 1996).

The antisense construct did not result in any resistance except for one line, 425-1, that showed deleyed symptom development. Other workers have also found antisense RNA-mediated resistance to plant RNA viruses to be ineffective (Smith *et al*., 1995). This is because antisense RNA-mediated resistance is thought to act in the cytoplasm where the transgene will be too dilute to work effectively by mechanisms such as hybridization.

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 hybridization 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.

5.8 Cowpea transformation

The sectored appearance of GUS positive plants was expected since the transformation procedure targets the general apical meristem area of the cowpea seedling. As a result, both meristematic and somatic cells may become transformed, to result in a chimeric plant. Such a chimeric plant appears as a mosaic of transformed and non-transformed sectors, and poses a challenge in terms of sampling especially in this particular case where a destructive GUS assay was used. Since PCR is very sensitive and amplifies any signal present, the CP transgene could be detected in some GUS positive plants. However, the signal detected by both the GUS assay and PCR could be transient, and Southern analysis is the standard way of determining whether integration has occurred. Only one plant gave a positive hybridization signal in Southern analysis. Further

analysis of this line through subsequent generations, if fertile, would be necessary. However, there is need to ensure that the germline is transformed to enable the transgene to be passed to subsequent generations.

The GUS positive sectors were obtained only from plants that had cotyledons attached, open first true leaves and had developed straight stems at the time of manipulation. The electrotransformation procedure stresses the seedling, and only those seedlings that have developed sufficiently will take up exogenous DNA, survive and develop using the food reserve of cotyledons as well as the photosynthate from first true leaves.

The pBI121 binary constructs used in this experiment have a gene for kanamycin resistance. However, kanamycin resistance is not an effective assay against germinating cowpea seedlings since the germinating cowpea seedlings are not affected by kanamycin. This is probably because of the large food reserves of the seedlings.

The various seedling pre-treatments except punching the meristem did not appear to improve transformation efficiency. Punching the meristem wounds the seedling and may make the meristematic cells more accessible to the exogenous DNA since the epidermal cells will have been removed. Acid and calcium chloride pretreatments were expected to make the cell wall and cell membrane respectively more permeable to DNA. Besides chemically weakening the cell wall, acid pretreatment may also induce the production of expansins that may result in further weakening of cell walls (Cosgrove, 2001). The heat and plant growth substance pretreatments were expected to induce other chemical messengers and heat shock proteins that may increase the chances of integration events in the cell (Hong & Verling, 2001). However, no improvement in transformation efficiency was observed.

The mechanism of DNA integration after uptake by electrophoresis is not known, but is likely to occur by non-homologous recombination into sites on the genome that are undergoing repair or replication, as is the case for other direct DNA transfer methods (Smith *et al*., 2001). It is not surprising that not all GUS-positive lines tested CP-positive (Plant 213, for instance). This also means that it is possible that some transformants were GUS-negative but CP-positive, and these were not detected in this screening procedure.

Transformation by electrophoresis, if successful, is a procedure that can be used to avert one of the major concerns of GMOs. The procedure does not require the use of selectable markers such as antibiotic or herbicide resistance genes, and only the exact sequence required for a particular characteristic in the transgene may be used. It is not understood how integration would occur, but T-DNA borders do not seem to be required. Under the conditions tested so far, integration appears to be random, and is not enhanced by pre-treatment with high temperature, hydrochloric acid, calcium chloride, kinetin, BAP or NAA. Both circular and linearised DNA seem to be effective. However, the seedling must have developed a straight stem with the first true leaves open, but the cotyledons must be intact. This may be important in ensuring survival of the seedling after the rather harsh handling and subjection to electrophoresis that stresses the plant.

6 CONCLUSIONS AND RECOMMENDATIONS

Cowpea aphid-borne mosaic virus isolate 3 (CABMV-Z3) infects *N. benthamiana*, *N. clevelandii*, and *Vigna unguiculata*, *Ch. quinoa* and *Ch. amaranticolor*, resulting in observable symptoms, but does not infect *N. glutinosa*, *N. tabaccum* (cv. White Burley), *N. rustica*, *Phaseolus vulgaris*, and *Cucumis sativus.* *Chenopodium amaranticolor* is a good local lesion host of the virus. High titre polyclonal antiserum against CABMV-Z3 and a full-length clone of CABMV-Z3 were prepared. These are invaluable tools that can be used in the study and monitoring of this virus that can potentially wipe out an entire cowpea crop.

Coat protein-mediated resistance to CABMV as evaluated in *N. benthamiana* results in delay in symptom development and tolerance. This is significant resistance since it allows the plants to grow to maturity and produce seed. Antisense-mediated resistance is not very effective. RNA-mediated resistance as conveyed by the pBI121-CP_{stop} construct in this investigation is the best strategy since it results in delayed symptom development, tolerance and recovery. However, the lines showing RNA-mediated resistance need to be investigated further to determine the steady-state RNA levels and presence of 21-25nt long RNA fragments characteristic of PTGS and RNA silencing mechanisms.

Cowpea transformation by electrophoresis results in low efficiency of transformation, and the transformants obtained so far are chimeric. The system still requires further optimisation in terms of targeting of meristematic cells and determination of treatments that might increase the chances of integration into germline cells. Efforts to further develop this method of plant transformation should continue because it has the potential to deliver the gene of interest, free of marker genes and any other unwanted sequences, to the genome of a target plant.

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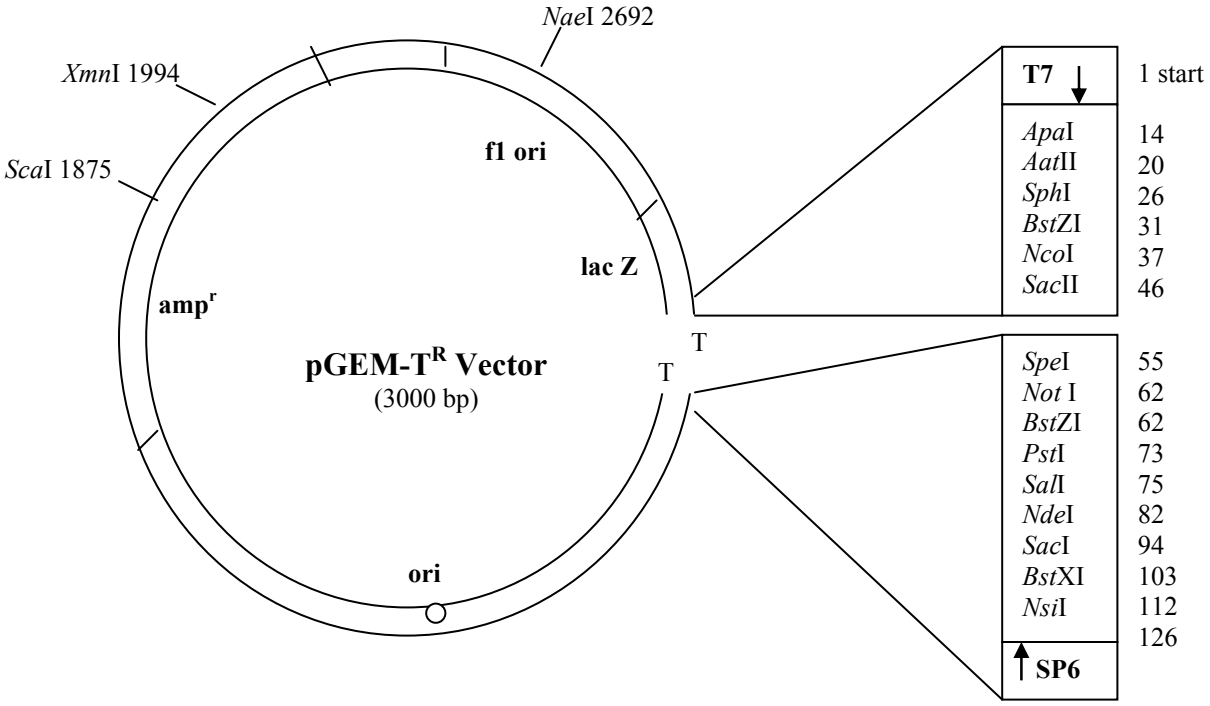
8 APPENDICES

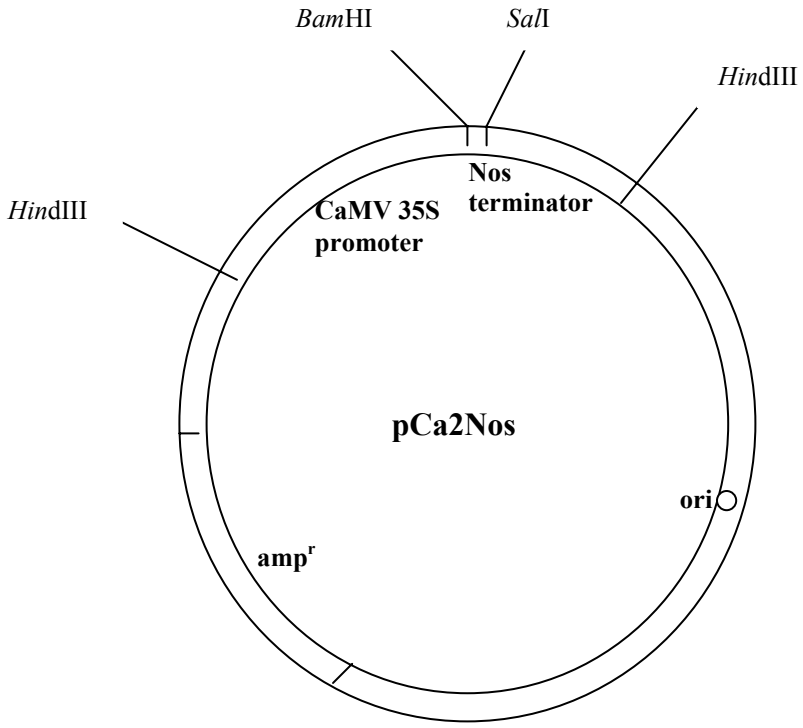
APPENDIX A

Plasmid vector map of pGEM-T



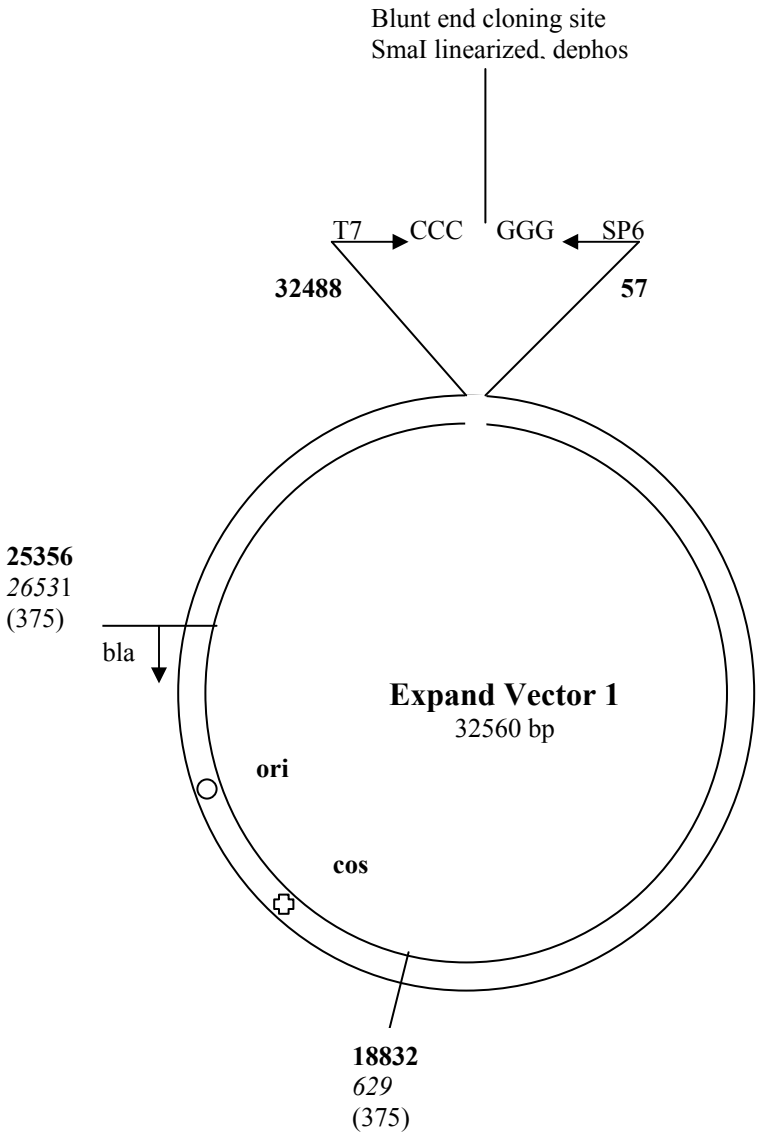
APPENDIX B

Plasmid vector map of pCa2Nos



APPENDIX C

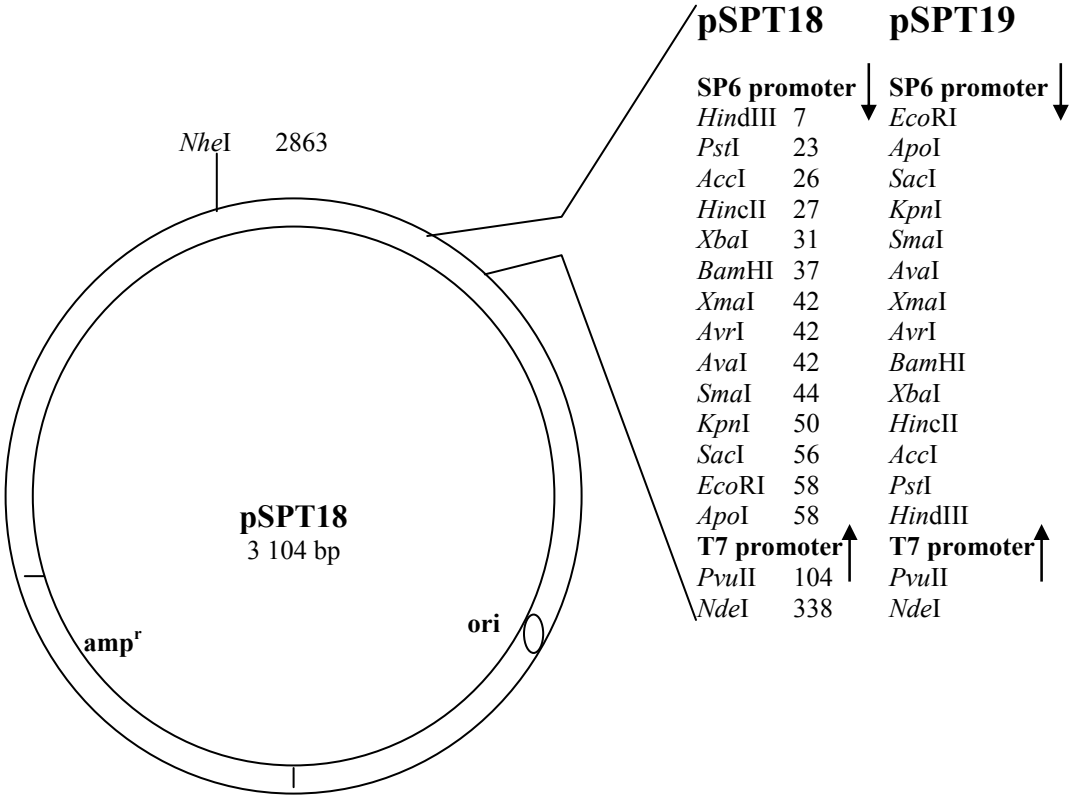
Plasmid vector map of pEV1



Legend:
Position in bold e.g. **25356** = Expand™ Vector
Position in italics e.g. *26531* = λ Stuffer Vector
Position in brackets e.g. (375) = pHC79
Dark part of the vector = pHC79, Bright part of the vector = λ Stuffer

APPENDIX D

Plasmid vector map of pSPT18/19



APPENDIX E

MGL Medium

5g mannitol

1g L-glutamic acid

1.15g sodium glutamate

0.25g H_2PO_4

0.1g NaCl

0.1g $MgSO_4.7H_2O$

1µg Biotin

5g Tryptone

2.5g Yeast Extract

Adjust pH to 7.0 with sodium hydroxide.

APPENDIX F

Abbreviations for names of viruses

ACMV – African cassava mosaic bigemini virus

AMV – Alfalfa mosaic alfamovirus

BaYMV – Barley yellow mosaic bymovirus

BCMV – Bean common mosaic potyvirus

BCTV – Beet curly top hybrigeminivirus

BDMV – Bean golden mosaic bigeminivirus

BGMV – Bean golden mosaic bigeminivirus

BlCMV – Blackeye cowpea mosaic virus

BMV – Brome mosaic potyvirus

BPMV – Bean pod mottle comovirus

BYDV – Barley yellow dwarf luteovirus

CABMV – Cowpea aphidborne mosaic virsus

CaMV – Cauliflower mosaic caulimovirus

CCMV – Cowpea chlorotic mottle bromovirus

CGMV – Cassava green mottle nepovirus

CIRV – Carnation Italian ringspot tombusvirus

CMMV – Cocksfoot mild mosaic sobemovirus

CMoV – Carrot mottle umbravirus

CMV – Cucumber mosaic cucumovirus

CTV – Citrus tristeza closterovirus

CYMV – Cacoa yellow dwarf mosaic virus

CyRSV – Cymbidium ringspot tombusvirus

CYVV – Clitoria yellow vein tymovirus

GCMV – Grapevine chrome mosaic nepovirus

GFLV – Grapevine fanleaf nepovirus

LMV – Lettuce mosaic potyvirus

MCMV – Maize chlorotic mottle machlomovirus

PCSV – peanut chlorotic streak caulimovirus

PEBMV – Pea early browning tobravirus

PLRV – Potato leafroll luteovirus

PMMV – Pepper mild mosaic potyvirus

PRSV – papaya ringspot potyvirus

PSbMV – Pea seed-borne mosaic potyvirus

PStV – peanut stripe potyvirus

PVA – Potato A potyvirus

PVM – Potato M carlavirus

PVS – Potato S carlavirus

PVS/PMV – Potato S carlavirus

PVX – Potato X potexvirus

PVY – Potato Y potyvirus

RMV – Ribgrass mosaic tobamovirus

RSV – Rice stripe tenuivirus

SAPV – South Africcan passiflora virus, renamed CABMV-SAP

SBMV – Bean southern mosaic sebamovirus

SHMV – Sunn-hemp mosaic tobamovirs

TBSV – Tomato bushy stunt tombusvirus

TEV – tobacco etch potyvirus

TGMV – Tomato golden mosaic bigeminivirus

TMGMV – Tomato mild green mosaic tobamovirus

TMV – Tobacco mosaic tobamovirus

ToMoV – Tomato mottle bigeminivirus

ToMV – Tomato mosaic tobamovirus

ToRSV – Tomato ringspot nepovirus

TRSV – tobacco ringspot nepovirus

TRV – Tobacco rattle tobravirus

TSMV – Tomato spotted wilt tospovirus

TSV – Tobacco streak virus

TSWV – Tomato spotted wilt tospovirus

TuMV – turnip mosaic potyvirus

TVMV – tobacco vein mottling potyvirus

TYLCV – Tomato yellow leaf curl bigeminivirus

WCMV – white clover mosaic potex virus

WSMV – Wheat streak mosaic rymovirus

ZYMV – Zucchini yellow mosaic potyvirus