The development of transgenic plants resistant to cucumber mosaic virus and tobacco necrosis virus

by

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Dedicated to my parents Daryl and Priscilla Hackland,

my sister Sandra and

to the late Philip Lloyd, an excellent friend and great climbing partner.

The development of transgenic plants resistant to cucumber mosaic virus and tobacco necrosis virus

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ABSTRACT

Cucumber mosaic virus (CMV) and tobacco necrosis virus (TNV) often occur in mixed virus infections in South Africa. Both viruses are of economic importance because of their world-wide distribution, extensive host range and their effects on yields of agriculturally important crop plants. The complete cDNA sequences of CMV-Wemmershoek (CMV-Wem) coat protein (CP) and TNV-F5P CP genes were cloned and subjected to sequence analysis. CMV-Wem is closely related to CMV-WL and CMV-Q, and therefore falls into CMV subgroup II. Similar analysis showed that TNV-F5P is closely related to TNV-A. By characterizing and sequencing these clones the authenticity of the CMV and TNV CP genes was also determined, prior to subcloning into the appropriate vectors for expression in *E. coli* and tobacco.

Constructs containing both the full-length CP genes of CMV-Wem and TNV-F5P were subcloned in frame with the *malE* gene, encoding the maltose binding protein (MBP), in the IPTG-inducible pMAL™ vector system, and expressed in *E. coli*. Through immunological detection the authenticity of both CPs was confirmed. The CMV CP translation product expressed in *E.coli* was used as an antigen to raise antiserum free from contaminating plant host-specific antibodies.

The CP genes of both viruses were individually cloned in both orientations (sense and antisense) in *Agrobacterium tumefaciens* Ti-plasmid-based binary and cointegrate vectors. The study was then extended to include engineering doubly transgenic plants. In order to determine whether the full-length CP is required to mediate virus resistance, a truncated form of the TNV CP was generated by deleting 83 amino acids from the C-terminus. Transgenic *Nicotiana tabacum* cv Petit Havana SR1 plants containing one of a number of different forms of CMV and TNV CP nucleotide sequence were generated. In whole plant studies, mechanical inoculation of R_o lines with CMV-Wem resulted in more than 50% of the CMV CP-sense (CP+) and CP-antisense plants not developing visible systemic disease symptoms. In both the CMV CP+ and doubly transgenic plants CMV-Wem accumulation was delayed, but virus was found to accumulate in the inoculated leaves over time. The CMV CP+ lines showed excellent protection against CMV-Q, but showed only a delay in symptom prodution when inoculated with CMV-Y, from subgroup I.

Mechanical inoculation of transgenic plants expressing either the full-length TNV-CP, the CP antisense transcript or a truncated form of the CP, showed an overall reduction in necrotic lesions. Symptom phenotype was altered, ranging from near wild type susceptibility, in some cases, to apparent immunity. It appears that expressing a dysfunctional CP in transgenic plants is an effective means of mediating virus resistance. The results obtained suggest that there is possibly more than one mechanism involved in the resistances observed. A proposal is made to account for these observations.

CHAPTER 1

Literature review: coat protein-mediated resistance in transgenic plants

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CHAPTER 1

Literature review: coat protein-mediated resistance in transgenic plants

1.1 INTRODUCTION

In this chapter I will first describe the proposed mechanism(s) of classical virus cross-protection in plants, followed by those suggested for coat protein-mediated resistance (CP-mediated resistance). Although both have common features, cross-protection is thought to be a complex response caused by the replication and expression of the entire viral genome, whereas the resistance conferred by the expression of a virus coat protein gene is more limited. The term genetically engineered cross-protection is frequently used because in many cases the phenotype of resistance mimics that of cross-protection. However, CP-mediated resistance, although a narrow term, more accurately describes the resistance that results from the expression of a virus CP gene in transgenic plants.

1.1.1 Classical cross-protection: proposed mechanisms

The natural phenomenon of viral cross-protection was first described by McKinney (1929), who showed that infection of a host plant with a mild strain of tobacco mosaic virus (TMV) protected the plant against subsequent superinfection by severe strains of the same virus. Cross-protected plants may have a reduced number of infection sites following challenge inoculation, may exhibit a distinct delay in symptom development, or may show no signs of the secondary viral infection. Cross-protection has been used to investigate relationships among viruses, and has been shown to be most effective against "closely related" strains, but not against distantly related viruses or virus strains (Wen *et al.*, 1991).

Cross-protection involves an interaction or interactions between related virus strains, and may therefore encompass recognition and signalling events between viral RNAs or virus coded proteins. The events that occur in the host that control the outcome of the interaction between virus strains have yet to be fully

explained. This has resulted in the generation of a number of hypotheses to attempt to explain the phenomenon. These are discussed individually, but combinations of each may contribute to the overall mechanism of cross-protection.

The first involves the annealing of sense and antisense RNAs of the inducing and challenge virus to prevent replication and/or translation of the challenging strain. In an early study Zaitlin (1976) demonstrated the possible role of RNA:RNA interactions, using a TMV strain that encoded a CP incapable of encapsidating its viral RNA. It was suggested that cross-protection resulted from intermolecular hybridization between the RNA of the protecting virus and nascent antisense strands on the replicative intermediate molecules of the challenge virus.

The second hypothesis proposes the re-encapsidation of the challenging viral RNA by free CP of the protecting strain, or blockage of uncoating. Several studies suggest that the CP plays a major role in cross-protection. About the same time that Zaitlin (1976) proposed the model of RNA:RNA interactions described above, de Zoeten and Fulton (1975) proposed that cross-protection occurs when RNA of the challenge virus is re-encapsidated in the CP produced by the protecting strain. This hypothesis is somewhat clouded by the fact that the cross-protection can, in some cases, be overcome when the challenge inoculum is viral nucleic acid rather than virions. This is the case for TMV (Sherwood and Fulton, 1982) and cucumber mosaic virus (CMV) (Dodds *et al.*, 1985).

A number of people have attempted to rationalise the role of the CP in this proposed mechanism of cross-protection. Sherwood and Fulton (1982) suggested that the challenge virus is unable to uncoat when inoculated onto plants systemically infected with the protecting strain. Wilson (1984) showed that TMV is disassembled cotranslationally by ribosomes *in vitro*. Wilson then suggested that free CP subunits from TMV may inhibit the disassembly process. This was subsequently found to be so, and Wilson and Watkins (1986) concluded that when parental virus particles from the challenge inoculum encountered high levels of free CP, the partial uncoating of the 5' end of the incoming (challenge) virus was prevented. This in turn blocked ribosome initiation and subsequent uncoating.

To detect inhibition of superinfection by CP, a challenge virus or its RNA must induce lesions in the protected leaves. The effect of CP on superinfectivity can then be measured by encapsidating the successful challenge RNA in various CPs, and assaying the infectivity of the virions in healthy and protected leaves. Zinnen and Fulton (1986) carried out such cross-protection studies with two Tobamoviruses, sunn hemp mosaic virus (SHMV) and TMV. When plants infected with SHMV were challenged with TMV RNA encapsidated in either SHMV CP or TMV CP, the RNA encapsidated in SHMV CP was 5-27 times less infectious than when it was encapsidated in TMV CP. This suggested that free homologous protein could diminish infectivity by inhibiting viral uncoating. These results in plants are consistent with the predictions derived from tests *in vitro* (Wilson and Watkins, 1986), but it is clear that other factors may be involved.

Interestingly, in contrast to the above hypothesis, Sarkar and Smitamana (1981) and Gerber and Sarkar (1989) have shown that CP-free mutants of TMV also induce cross-protection. In addition it has been shown that plants infected with tobacco rattle virus RNA 1 (which does not encode the CP) are protected against subsequent inoculation with strains of this virus (Cadman and Harrison, 1959). This led to the conclusion that factors other than encapsidation and the blocking of uncoating of the challenger are at work.

The third major hypothesis suggests that there is competition between the protecting strain and the challenge virus for a factor present in the host cell, for example the replicase components. Gibbs (1969) suggested that the nucleic acid of the challenge virus could irreversibly bind to the replicase of the virus already present in the cell if the challenge virus had a similar, but not identical, replicase recognition site. However, a viral nucleic acid with either an identical or very different replicase recognition site could replicate and be translated unhindered. The production of pseudorecombinants of raspberry ringspot virus (RRV) (Harrison *et al.*, 1974) and other viruses demonstrates that related strains of a virus can utilize the same replicase. Thus the replicase may in some way be involved in cross-protection. Horikoshi *et al.* (1987) showed a distinct involvement of the CP of brome mosaic virus (BMV) with the replicase. They found through *in vitro* studies that the BMV CP blocked the binding site of the replicase, thereby interfering with RNA synthesis. They suggested that the regulation of replication by CP may be the basis of cross-protection, and this

would therefore not require the complete encapsidation of the RNA of the challenge virus.

It is evident from the breadth of observations on the subject of cross-protection that comparisons between different studies are difficult as each study has a different set of parameters and factors which influence the findings. Several mechanisms may be involved in the protection afforded by the resident virus against superinfection by another virus or strain. In the initial interaction the superinfecting virus could be inhibited from uncoating, thereby preventing initiation of the replication cycle. If replication is initiated, a number of mechanisms may be involved in the control of the replication of the superinfecting RNA virus: (1) the initial translation of the incoming (+)RNA could be blocked, (2) the transcription of the incoming (+)RNA could be prevented even if it is initially translated, and (3) the production of nascent (+)RNA strands from (-)RNA could be inhibited. Several of the proposed mechanisms could account for these events.

In order to reconcile the data into a single hypothesis researchers were and still are left to identify mutants which lack a function associated with a specific gene product and then to correlate this loss with a loss of protection. Obviously mutants which have lost the ability to replicate cannot be analyzed for cross-protection. In addition no study has provided a model which could analyse the role of each viral product, for example viral nucleic acid and viral coat protein, in cross-protection. An experimental system is therefore required which could separate the different aspects of this complex interplay of factors.

1.2 GENETIC TRANSFORMATION TO PRODUCE VIRUS RESISTANT PLANTS

The tools of molecular biology and genetic transformation allow expression of fragments of the viral genome throughout transgenic plants, or in specific tissues. The ability of these fragments to provide protection can then be evaluated. In the mid-1980s Sanford and Johnston (1985) proposed a broadly-applicable strategy for genetically engineering resistance to parasites. They stated: "It is rare for a useful strategy to be conceived of by man which is not already operational somewhere in nature." As has been described, closely related plant viruses, or different strains of the same virus, will cross-protect, and therefore it is likely that in these cross-protected plants a direct form of parasite-

derived resistance occurs. Engineering this parasite-derived resistance has been considerably more approachable on the molecular level than the engineering of host-derived resistance.

1.2.1 Expression of viral CP coding sequences in transgenic plants

The first published report of the expression of viral cDNAs in transgenic plants described the expression of sequences of TMV RNA that encoded the CP (Bevan *et al.*, 1985). In this report a chimaeric construct was made containing the CP gene of the OM strain of TMV flanked by the 35S RNA promoter (35S promoter) from cauliflower mosaic virus (CaMV) and the polyadenylation signal from the *Agrobacterium tumefaciens* nopaline synthase (*nos*) gene. Tobacco plants regenerated from transformed leaf pieces expressed the chimaeric gene. Plants accumulated CP to the level of 0.001% (w/w) of total soluble leaf protein. However when R1 progeny of the transgenic plants were inoculated with TMV they were found to be as susceptible to infection as the non-transgenic controls.

Powell-Abel *et al.*, (1986) expressed the CP gene of the U_1 strain of TMV in transgenic plants and found that TMV-CP accumulated to a level of 0.1% (w/w) of total soluble protein from transgenic leaf tissue. This was some 20-100X higher than the levels of those reported by Bevan *et al.* (1985). The seedlings that expressed the CP gene exhibited delayed symptom development after viral challenge and 10% to 60% of the transgenic plants failed to develop symptoms. This was the first indication that plants could be genetically transformed for resistance to virus disease development.

There are a number of possible reasons for the differences in levels of accumulated CP in the transgenic plants produced by the two groups. The two laboratories used different DNA fragments to provide the 35S promoter, which may have resulted in different rates of transcription. Differences in the untranslated sequences 5' of the ATG initiation codon of the CP coding sequences may control the rate of translation of the transcript: Rogers *et al.* (1985) reported that additional ATG codons in this untranslated leader sequence can lower the level of protein synthesis from mRNA in plant cells. Bevan *et al.* (1985) used a construct which contained an out of frame ATG 5' of the CP coding sequence. In addition, differences in 3' untranslated sequences may contribute to the differences in stability or translatability of the transcripts in the two experimental systems. It is obvious from just these two examples that numerous

factors may determine the outcome of incorporating and expressing viral genes in plants.

1.2.2 Examples and phenotypes of CP-mediated resistance

Since the first report of CP-mediated resistance against TMV in tobacco, there have been an increasing number of examples of resistance due to CP gene expression. The resulting resistance has also often proved to be effective in a number of field trails. A summary, presented in Table 1.1., shows that CP-mediated resistance is effective against viruses in 14 different virus taxonomic groups, in a number of different crop plants.

The incorporation and expression of virus capsid protein genes has provided one of the strongest forms of cross-protection or virus resistance yet developed. In each example of CP-mediated resistance described to date, resistance is manifested by several features. However not all of these examples exhibit each of the phenotypes described below.

Firstly, the number of sites where infection occurs on inoculated leaves may be reduced: Loesch-Fries *et al.* (1987) and Hemenway *et al.* (1988) reported fewer lesions on AlMV CP(+) and PVX CP(+) plants following inoculation with AlMV and PVX, respectively, than on the non-transgenic control plants.

A second manifestation of resistance may be a delay or absence of systemic disease development through the CP(+) plants. Hemenway *et al.* (1988) reported that the systemic infection in CP(+) plants was delayed in response to RNA inoculation, and the extent of protection observed was similar to that seen in response to virus inoculation.

A third manifestation of resistance may be a reduction in virus accumulation in infected CP(+) plants compared to infected non-transgenic plants. Virus accumulation in inoculated leaves and other plant parts has been quantified by enzyme-linked immunoadsorbent assay (ELISA) or by western blot analysis. Transgenic plants that expressed the CP of CMV, PVX and potato virus Y (PVY) accumulated much less virus, if any at all in some cases, following inoculation with the respective viruses (Cuozzo *et al.*, 1988; Lawson *et al.*, 1990; Hemenway *et al.*,1988)

TABLE 1.1 Summary of Examples of Coat Protein-Mediated Resistance[a]

Virus Group	CP Gene	Chimeric CP construct	Transgenic Plant	Challenge Virus/Group	CP-MR[b]	References
Cucumo	CMV-C	P35S[1]:CMV:rbcS-E9[2]	Tobacco	CMV-C/Cucumo		Cuozzo *et al.*, 1988
	CMV-WL	P35S:CMV:35S-3'UT	Tobacco	CMV-WL		Namba *et al.*, 1991
				CMV-Chi		
				CMV-C		
	CMV-O	P35S:CMV:NOS[3]	Tobacco	CMV-O		Nakajima *et al.*,1993
				CMV-Y		
				CMMV/Cucumo		
	CMV-Y	P35S:CMV:35S-3'UT	Tobacco	CMV-Y		Okuno *et al.*, 1993
Alfamo Mosaic	AlMV	P19S:AlMV:CaMV	Tobacco	AlMV/Alf Mosaic		Loesch-Fries *et al.*, 1987
		P35S:AlMV:NOS	Tobacco	AlMV		Tumer *et al.*, 1991
		P35S:AlMV:rbcS-E9				
		P35S:AlMV(mut)[4]:rbcS-E9			No	
		P35S:AlMV:NOS	Tomato	AlMV		Tumer *et al.*, 1987
						van Dun *et al.*,1987
		P35S:AlMV:T-DNA ORF25	Tobacco	AlMV		Halk *et al.*, 1989
		P35S:AlMV:T-DNA ORF25	Alfalfa	AlMV		Hill *et al.*, 1991
		P35S:AlMV(mut):NOS	Tobacco	AlMV	No	van Dun *et al.*, 1988
Ilar	TSV	P35S:TSV:NOS	Tobacco	TSV		van Dun *et al.*, 1988
				AlMV	No	
Potex	PVX	P35S:PVX:rbcS-E9	Tobacco	PVX/Potex		Hemenway *et al.*, 1988
		P35S:PVX:NOS	Potato	PVX		Hoekema *et al.*, 1989
		eP35S[5]:PVX:rbcS-E9	Potato	PVX		Lawson *et al.*, 1990
		[6]Extensin:PVX:NOS	Potato	PVX		Fehér *et al.*, 1992
Poty	PVY	eP35S:PVY:rbcS-E9	Potato	PVY/Poty		
	SMV	P35S:SMV:NOS	Tobacco	SMV/Poty		Stark and Beachy, 1989
				PVY		
				TEV/Poty		
	PRV	P35S:PRV:NOS	Papaya	PRV/Poty		Fitch *et al.*, 1992
			Tobacco	PRV		Ling *et al.*, 1991
				TEV		
				PVY		
				PeMV		
				CMV/Cucumo	No	
	PPV	P35S:PPV:35S-3'UT	Tobacco	PPV		Regner *et al.*, 1992

(Continued)

TABLE 1.1 **Summary of Examples of Coat Protein-Mediated Resistance**[a]

Virus Group	CP Gene	Chimeric CP construct	Transgenic Plant	Challenge Virus/Group	CP-MR[b]	References
	ZYMV	P35S:ZYMV:35S-3'UT	Muskmelon	ZYMV/Poty		Fang and Grumet, 1993
Tobamo	TMV	P35S:TMV:NOS	Tobacco	TMV/Tobamo		Powell-Abel *et al.*, 1986
		P35S:TMV:NOS	Tobacco	ToMV/Tobamo		Nejidat and Beachy, 1990
				TMGMV		
				ORSV		
				RMV	No	
				PMMV		
				SHMV	No	
		P35S:TMV:NOS	Tomato	TMV		Nelson *et al.*, 1987
				ToMV		
	ToMV	eP35S:ToMV:NOS	Tomato	ToMV		Sanders *et al.*, 1992
Luteo	PLRV	[7](P35S)[2]:PLRV:NOS	Potato	PLRV/Luteo		Kawchuk *et al.*, 1990/1991
		P35S:PLRV:NOS	Potato	PLRV		van der Wilk *et al.*,1991
		P35S:PLRV:NOS	Potato	PLRV		Barker *et al.*, 1992
Tobra	TRV	P35S:TRV:NOS	Tobacco	TRV-TCM		van Dun *et al.*, 1987
				TRV-PLB	No	van Dun and Bol, 1988
				PEBV/Tobra		
Carla	PVS	(P35S)[2]:PVS:NOS	Potato	PVS/Carla		MacKenzie *et al.*, 1991
				PVM		
Tospo	TSWV	(P35S)[2]:TSWV:NOS	Tobacco	TSWV/Tospo		MacKenzie and Ellis, 1992
Nepo	GCMV	P35S:GCMV:NOS	Tobacco	GCMV/Nepo		Brault *et al.*, 1993
	ArMV	eP35S:ArMV:NOS	Tobacco	ArMV		Bertioli *et al.*, 1992
Tenui	RSV	P35S:RSV:NOS	Rice	RSV/Tenui		Hayakawa *et al.*, 1992
Furo	BNYVV	P35S:BNYVV:NOS	Sugarbeet	BNYVV/Furo		Kallerhoff *et al.*, 1990
Tombus	CyRSV	P35S:CyRSV:NOS	Tobacco	CyRSV/Tombus		Rubino *et al.*, 1993

[a]List of abbreviations: AlMV, Alfalfa mosaic virus; ArMV, Arabis mosaic virus; BNYVV, Beet necrotic yellow vein virus; CaMV, Cauliflower mosaic virus; CMV, Cucumber mosaic virus; CMMV, Chrysanthemum mild mottle virus; CyRSV, Cymbidium ringspot virus; GCMV, Grapevine chrome mosaic virus; ORSV, Ondontoglossum ringspot virus; PEBV, Pea early browing virus; PeMV, Pepper mottle virus; PLRV, Potato leaf roll virus; PMMV, Pepper mild mottle virus; PPV, Plum pox virus; PRV, Papaya ringspot virus; PVM, Potato virus M; PVS, Potato virus S; PVX, Potato virus X; PVY, Potato virus Y; RMV, Ribgrass mosaic virus; RSV, Rice stripe virus; SHMV, Sunn hemp mosaic virus; SMV, Soybean mosaic virus; TEV, Tobacco etch virus; TMGMV, Tobacco mild green mosaic virus; TMV, Tobacco mosaic virus; ToMV, Tomato mosaic virus; TRV, Tobacco rattle virus; TSV, Tobacco streak virus; TSWV, Tomato spotted wilt virus; ZYMV, Zucchini yellow mosaic virus.

[b]CP-MR: CP-mediated resistance. Positive (Yes) unless otherwise stated
[1]P35S: 35S promoter derived from cauliflower mosaic virus
[2]rbcS-E9: ribulose bisphosphate carboxylase terminator
[3]NOS: nopaline synthase terminator
[4](mut): mutant CP gene
[5]eP35S: enhanced P35S
[6]Extensin: extensin-gene promoter
[7](P35S)[2]: dual P35S

The final manifestation may be a reduction in severity of disease symptoms in plants that become infected. From the standpoint of complete disease control, it would be desirable for CP-mediated resistance to encompass each of the phenotypic traits described above. A reduction in severity of disease symptoms occurs in most, but not all cases of CP-mediated resistance.

All the different manifestations of resistance can usually, but not always, be overcome by inoculating with increasing concentrations of virus (Powell-Abel *et al.*,1986). Powell-Abel *et al.* (1986) and Loesch-Fries *et al.* (1987) also reported that inoculation of CP-expressing plants with TMV RNA and AlMV RNA respectively, overcame the protection. In contrast to this Hemenway *et al.* (1988) found that inoculation of plants expressing high levels of PVX CP with PVX RNA did not overcome the protection. Perhaps PVX CP can moderate early events in RNA infection by a different mechanism. This and other features of CP-mediated protection are described and discussed in the following sections.

1.2.3 Understanding the mechanisms of CP-mediated resistance

In the following section I will describe the observations and suggested proposals to account for CP-mediated resistance. It is clear that several mechanisms may be involved in the specific resistance resulting from the expression of a particular CP in plants. These mechanisms could operate at the initial interaction between the CP(+) plant and the challenge virus; or if replication is initiated, resistance mechanisms could involve the control of this replication or a later stage during viral movement. For this reason I have divided this section into two parts. The first deals with the proposed "inhibited-uncoating" hypothesis, an early event which occurs prior to replication of the challenge virus. The second part describes the proposed role of the expressed CP in the delay of disease development and systemic spread.

Early events in infection

Infection starts upon the introduction of a typical plant virus into its host with the release of the nucleic acid for translation by host ribosomes. Wilson (1985) proposed that for most viruses, swelling of the particle precedes the release of

the nucleic acid (see Mundry *et al.*, 1991). This is followed by binding of ribosomes and disassembly of the virus concurrent with translation. The question is, does the CP interfere in some way with this process, or is the interference due to accumulation of CP mRNA which hybridizes to viral RNA replication intermediates?

Several different findings indicate that CP gene expression interferes with an early event in infection. In the early protection studies it was found that resistance could be overcome by inoculation with viral RNA (TMV, Nelson *et al.*, 1987; AlMV, Loesch-Fries *et al.*, 1987; TSV, van Dun *et al.*, 1988). This indirectly showed that RNA:RNA interactions between the transgenic CP transcript and the challenge virus RNA were not providing much protection against primary infection.

To address the issue of the possible involvement of TNV CP-specific mRNA in providing protection, Powell *et al.* (1990) generated a construct that would produce untranslatable mRNA and a construct which encoded an mRNA that lacked the replicase binding site, but would translate into CP. The results indicated that only plants that accumulated CP, rather than CP RNA sequences, were protected against TMV infection.

These results confirmed the conclusions of Nejidat and Beachy (1989), who examined the levels of TMV CP in transgenic tobacco plants under different temperature conditions and analysed the effect of these treatments on the efficiency of CP-mediated protection. Interestingly, continuous high temperatures resulted in a decline in CP accumulation, but not in accumulation of CP mRNA. Under these conditions, a lower level of protection was observed. This implicates the CP and not the mRNA in protection. However protection was not found to decrease in tomato plants expressing the same gene under the same conditions. This indicates that we cannot ignore the potential differences in the host response (Nejidat and Beachy, 1989).

Studies with protoplasts have yielded important information about early events leading to CP-mediated resistance. The use of protoplasts makes it possible to distinguish between events that occur in individually infected cells and those that require the involvement of multiple cells. Furthermore these studies enable one to distinguish between molecular events that affect infection and/or replication, and exclude the movement of virus in the inoculated leaves through

the plant. Register and Beachy (1988) reported a decrease in the accumulation of viral CP and (+) and (-) strand viral RNA when TMV CP(+) protoplasts were challenged with TMV virions. There was however less resistance against TMV RNA and TMV treated at pH 8.0 (to swell the virions). This confirmed earlier results using whole plants (Nelson *et al.*, 1987). These experiments indicated that infection is blocked at a stage prior to the release of RNA from the virus and subsequent replication.

A number of elegant experiments were performed to test the "inhibited-uncoating" hypothesis. Osbourn *et al.* (1989a) encapsidated chimaeric reporter mRNA encoding β-glucuronidase (GUS) in TMV CP to form "pseudovirus" particles. When the TMV-like particles were introduced into protoplasts, they uncoated and expressed their nonreplicating "pseudo-genomes" transiently. The level of expression of the GUS reporter mRNA was a direct measure of the extent of pseudovirus disassembly. GUS-particles were expressed 100-fold less efficiently in CP(+) protoplasts, whereas unencapsidated GUS mRNA was expressed only 2.8-fold less efficiently. When TMV RNA was transcapsidated with heterologous CP *in vitro*, the hybrid virions were equally infectious in both the control protoplasts and protoplasts expressing the heterologous CP. Osbourn *et al.* (1989a) concluded that although compatible protein-protein interactions significantly inhibited the (GUS) nucleocapsid disassembly in CP(+) transgenic protoplasts, the endogenous CP must also interfere with a later stage of infection involving the viral RNA.

Wu *et al.* (1990) also examined the "inhibited-uncoating" hypothesis. Using radioactive TMV particles they were able to show that extracts of TMV CP(+) protoplasts infected with these particles contained fewer complexes thought to be involved in cotranslational disassembly of virus particles ("striposomes") than did extracts from non-transgenic protoplasts. This is direct evidence that the expressed CP inhibits the uncoating process and in turn the cotranslational disassembly mechanism. Osbourn *et al.* (1989b) tested the ability of TMV CP expressed in transgenic plants to encapsidate RNA. This was measured by introducing a second reporter gene whose transcripts contained a copy of the TMV origin-of-assembly sequence (OAS). Singly transformed TMV CP+ plants were used to create plants bearing both transgenes in which complementation between TMV CP and the OAS^+ mRNAs could be assessed. Packaging of the reporter mRNA was found to be insufficient to account for the "protective" action of the CP. They concluded that the endogenous CP must be more efficient at

preventing nucleocapsid disassembly or interfering with a later stage of virus replication (or both) than in packaging RNA from already uncoated (or partially uncoated) virus particles.

Another important question that needed to be answered was what effect the aggregation state of the expressed CP had on CP-mediated protection. Register and Beachy (1989) addressed this question and characterized the form of TMV CP which is responsible for protection. They found that extended helical aggregates of CP provided greater protection than did lower order structures (monomers to 20 S discs) in protoplast assays. It is well known that TMV CP particles can form quaternary structures in the absence of RNA (Butler, 1984).

Register and Beachy (1989) proposed that differences in levels of protection may result from differences between the intracellular localization of CP in transgenic plants and protoplasts. Wilson (1985) had already suggested that cellular membranes are involved in uncoating or destabilization of plant viruses and it is possible that the association of CP with membranes may be essential for protection against TMV infection. A question then arises, do the infecting TMV particles compete with higher order quaternary structures for cellular membrane binding sites in CP(+) plants, therefore reducing their chance of uncoating and replication? Interestingly, a novel feature of the transgenic plants which express arabis mosaic Nepovirus (ArMV) CP, is that the plants produce empty virus-like particles (Bertioli *et al.*, 1991). These authors proposed that it may be the pseudovirions themselves, or the CP in some different form, that mediates resistance (Bertioli *et al.*, 1992).

The above results do not change the perception of the principle role of the CP in binding to viral RNA *per se* and inhibiting uncoating. In fact it has been found that protection can also occur with heterologous proteins with an undetermined degree of amino acid sequence similarity, and it is clear that at least some form of protection is related to tertiary structure. A number of studies (Anderson *et al.* 1989; Stark and Beachy, 1989; Nejidat and Beachy, 1990) have attempted to expand the understanding of the breadth of protection conferred by CP genes. Stark and Beachy (1989) chose soybean mosaic Potyvirus (SMV), as it has a very limited host range. Plants accumulating SMV CP were found to be resistant to infection by two serologically unrelated Potyviruses. Since amino acid sequence homologies between the "protecting" SMV CP and those of the challengers tobacco etch virus (TEV) and potato virus Y (PVY) are relatively low (58% and

61%, respectively), the authors suggested that CP protection requires structural as well as sequence homology between the challenge virus and the protecting CP. Similarly, it was found that transgenic tobacco plants expressing the CP gene from a common strain of TMV are resistant to other Tobamoviruses whose CP amino acid similarity with the TMV CP is 67% to 87% (Nejidat and Beachy, 1990).

Quemada *et al.* (1991) showed that transgenic plants expressing the CP gene of CMV-C (subgroup I) are well protected against virus infections from the homologous subgroup. Within each subgroup, CP amino acid sequences share more than 95% sequence identity, while viruses in different subgroups share only 80% similarity. Interestingly Namba *et al.* (1991) showed that for the expression of the CMV-WL CP gene, the level of CP-mediated protection against heterologous CMV strains appears to be very similar to that found for the homologous CMV strains. Nakajima *et al.* (1993) have expressed CMV-O CP in transgenic tobacco and challenged these plants with a serologically unrelated Cucumovirus, chrysanthemum mild mottle virus (CMMV). These transgenic plants showed a significant level of protection against CMMV.

These results for Potyvirus, Cucumovirus and Tobamovirus CP protection demonstrate that CP-mediated protection is not necessarily a one CP gene/one virus phenomenon, but rather that the expression of a single CP gene can potentially protect a plant against several different viruses.

A suggestion was made by Nejidat and Beachy (1990) that 60% homology between the protecting CP expressed in the transgenic plants and the CP of the challenger virus provided sufficient conformational similarity for the protecting CP to block the uncoating process. However, until more definitive experiments are carried out it is difficult to conclude whether the overall amino acid sequence similarity of the "protecting" and challenge virus CPs, or sequence similarity within particular regions of the CP, or overall structure rather than sequence homology is important for protection. Tumer *et al.* (1991) demonstrated that the second amino acid residue of AlMV CP is critical for protection from AlMV virions and RNA. A CP mutated in this amino acid was still able to bind to the AlMV RNAs *in vivo*, activating replication, indicating that the mutation is not critical for this interaction. As proposed earlier partial capsid assembly may be a requirement for resistance. The mutant AlMV CP may not confer resistance as it is unable to undergo this partial assembly process.

In contrast, a few people have evidence against the above protein:RNA interaction in resistance (van Dun *et al.*, 1988): these authors reported that the CP of the tobacco rattle virus (TRV) TCM strain did not protect tobacco plants from infection by the PCB strain, although the CPs encoded by both strains could encapsidate the RNA molecules of the other strain. Also a number of contrasting and inconsistent results have been reported for CMV (Quemada *et al.* 1991; Namba *et al.* 1991; Nakajima *et al.* 1993). This suggests that no rule can be made for the spectrum of resistance delivered by a single expressed CP gene. Whether this inconsistency is going to be a common feature amongst all expressed CPs only time will tell. Interestingly Lindbo and Dougherty (1992) generated plants expressing the full-length TEV CP "sense" RNA molecule with a frameshift mutation rendering it untranslatable. These plants were found to be highly resistant to Potyvirus infection. In this case the CP is not present and the positive sense RNA is thought to interfere with transcription and/or translation, binding essential host or viral factors or interfering with virion assembly. There are a few reports in which transgenic plant lines are highly resistant to challenge inoculation, and yet the coat protein product is not observed (Kawchuk *et al.* 1990, 1991; van der Vlugt *et al.* 1992).

Later events: spread of infection

Another manifestation of protection is the delay of the spread of virus from inoculated leaves to upper leaves. During the course of a systemic viral infection in plants, viruses replicate and move short distances from cell to cell through plasmodesmata, and long distances through the vascular system. Cell-to-cell viral movement through the plasmodesmata requires viral-encoded movement proteins (MP) (reviewed by Hull, 1989). Less, however, is known about the long-distance movement: how the viruses enter, move through, and exit the vascular system. Viral genes and their products seem to be required for long-distance movement and are different from those required for cell-to-cell movement. Saito *et al.* (1989) showed that both the TMV CP, with an ability to assemble into virus particles, and the assembly origin in the TMV genomic RNA are involved in long distance movement. Mutations at certain positions of the CP or in the assembly origin of the TMV RNA greatly delay or abolish viral long-distance movement, while allowing cell-to-cell movement to proceed normally in the inoculated leaf. This suggests that with TMV whole viral particles may play a central role in long-distance movement. However, a single dogma concerning the mechanism of viral spread cannot be established. For instance, in the case of cowpea

chlorotic mottle Bromovirus there is a requirement for both the 3a movement protein and the CP for systemic infection (Allison *et al.*, 1990).

In early studies several groups reported a delay in development of systemic disease symptoms after inoculation of CP(+) transgenic plants with the respective viruses (Loesch-Fries *et al.*, 1987; Nelson *et al.*, 1987; van Dun *et al.*, 1987). This could result from interference with: (1) the spread of virus from cell to cell in inoculated tissue; (2) the movement of virus from the inoculated leaf into the vascular tissue; (3) movement through the vascular tissue, and (4) the movement into upper (or lower) non-inoculated leaves.

Grafting experiments were initiated by Wisniewski *et al.* (1990) to determine whether long-distance movement of TMV was reduced in CP(+) tissue compared with non-transgenic tissue. When stem sections grafted between the CP(-) root-stock and apical section were derived from a CP(+) plant line and contained a leaf, fewer plants developed disease symptoms compared with grafted plants that contained a CP(-) stem section. Interestingly, CP(+) grafted stem tissue without a leaf had no effect on TMV spread. A suggestion was made that perhaps the presence of the leaf tissue was acting as a virus sink. After entering the leaf these viruses may not be able to establish an infection. This would sufficiently reduce the virus titre and thus disable further spread.

It is now known that once viruses invade the vascular system in susceptible host plants they move in prescribed pathways. Cauliflower mosaic virus which moves systemically through phloem channels in plants such as turnip, is apparently swept along with the flow of photoassimilates from source leaves to sink leaves (Leisner *et al.*, 1993). Thus in systemic infections, young leaves import viruses from inoculated leaves, whereas mature leaves do not.

Wisniewski *et al.* (1990) also showed that the spread of TMV to closely adjacent tissue (1-3 mm) was similar in CP(+) and CP(-) plants, but spread to more distant tissues (5-10 mm) was significantly reduced. Considering that virus movement from cell to cell is via a riboprotein complex (Deom *et al.*, 1992), it may be that cells adjacent to those infected should become infected, as CP-mediated protection has been shown not to work when cells (protoplasts) or CP(+) transgenic plants are inoculated with the respective viral RNA or pH 8.0 treated particles (Nelson *et al.*, 1987).

In the case of TMV, the reduction in virus accumulation in systemically infected leaves was shown by Wisniewski *et al.* (1990) to be independent of the resistance that blocks initial infection, as it is not overcome by inoculation with TMV RNA. This is in contrast to an earlier theory of Dodds *et al.* (1985) who suggested that there may be a similar or identical mechanism preventing movement of infection within the plant as there is preventing initial infection.

Whatever the transport form, it is likely that the presence of CP in the phloem and associated cells interferes with viral long-distance spread and therefore with systemic disease development. This was confirmed by Clark *et al.*, 1990 who compared plant lines expressing similar levels of TMV CP from the *rbcS* and CaMV 35S promoters for resistance. In whole plant assays the 35S:CP constructs gave higher resistance than the rbcS:CP constructs. Since the CaMV 35S promoter is highly active in phloem-associated cells (Jefferson *et al.*, 1987), it is possible that plants expressing the 35S:CP gene inhibit virus loading into, movement through, or exit from, phloem and phloem-associated tissues to a greater extent than in rbcS:CP plants. These results clearly indicate that more than one mechanism exists in CP(+) plants, over and above those proposed mechanisms active during early events in virus infection, which provide further protection against virus multiplication.

A group of proteins postulated to be involved in plant virus resistance are the pathogenesis-related (PR) proteins, small extracellular plant polypeptides, whose synthesis is induced after pathogen infection. Van Loon (1985) showed these proteins to be associated with the hypersensitive response and/or resistance to systemic spread of TMV. Others have failed to observe this correlation and argued against their role in viral resistance (Bol *et al.*, 1987; Sherwood, 1985).

Bol (1988) speculated that a group of PR proteins induced by TMV infection may be cell wall components because of their high glycine content: that these proteins may alter the cell wall structure and subsequently affect virus movement. Cutt *et al.* (1989) constitutively expressed the PR1b gene in tobacco plants and found that the product was properly cleaved and secreted into the intercellular fluid of the transformed plants. Interestingly, they also demonstrated that expression of the PR1 gene caused no apparent delay in onset or alteration of systemic disease symptoms. Lowering the virus inoculum also proved unsuccessful in

demonstrating a low level of viral resistance. This suggests that the PR1b protein by itself does not possess antiviral activity. Therefore viral resistance is probably conferred by a factor(s) other than, or in addition to, single PR proteins.

We know that in some cases virus infection does stimulate the PR protein "defence mechanism". Could there be an association between the expression of CP in transgenic plants and the stimulation of synthesis of these potentially protective PR proteins, leading to enhanced virus resistance? Carr *et al.* (1989) answered this question by showing that PR accumulation was not enhanced in TMV CP(+) plants. The data presented suggested that CP-mediated resistance against viruses in CP-expressing transgenic plants is unlikely to involve PR proteins. However, no direct evidence has excluded this viewpoint, and no specific antiviral-function has been assigned to the PR proteins.

1.2.4 Levels of CP expression versus levels of resistance

If the expression of viral CP plays a role in protection of transgenic plants, one might expect that the level of expression in the plant may be important. In many cases documented to date it has been clear that plant lines with higher levels of expressed CP do in fact exhibit higher levels of resistance. This is not a general rule, however: in some studies there was a clear lack of correlation, and the levels of CP accumulation could not be used as a guide to selecting virus resistant plant lines. In these cases (Kawchuk *et al.*, 1990; Kawchuk *et al.*, 1991; van der Vlugt *et al.*, 1992) the resistance was associated with the positive sense CP RNA molecule and its interaction with virus or host functions. This phenomenon of resistance has been studied by introducing a CP gene that produces a transcript that is incapable of translation (Lindbo and Dougherty, 1992).

In almost all studies of this nature the promoter of choice has been the 35S promoter of CaMV and although this promoter is nominally constitutive, the pattern of expression can be different in different plants. In addition phenotypes can be governed by "when and where" the gene is expressed rather than simply "how much". Whether or not position effects control the efficacy of the CP to limit virus infection remains to be determined.

1.2.5 Expression of the antisense RNA of the viral CP in transgenic plants

It was established with bacterial systems that RNA-mediated functions could be inhibited if the cells were engineered to express the RNA complement (i.e. antisense RNA) of the biologically active form (Pines and Inouye, 1986). Sandler *et al.* (1988) reported the successful suppression of nopaline synthase (NOS) enzymatic activity in plants via the overexpression of antisense RNAs complementary to the *nos* mRNA. The same mechanism was expected to provide greater protection in plant RNA virus systems than (+) sense CP transcripts. In theory, the antisense RNA complementary to CP mRNA could hybridize to both the (+) sense genomic and subgenomic RNA thereby rendering them nonfunctional. Initially a number of studies using antisense gene constructs showed only minimal protection even at low virus inoculum levels (PVX, Hemenway *et al.*, 1988; CMV, Cuozzo *et al.*, 1988).

However, more recently a number of reports have shown that antisense RNA mediated protection should not be discounted as there is evidence to suggest that the different results are due to differences between the viruses and their modes of infection and replication, as well as differences in the host.

Potato leafroll Luteovirus (PLRV), unlike PVX and CMV, occurs at a low titre in plants and is confined to the phloem of the host plant. Kawchuk *et al.* (1991) used an antisense construct of the PLRV CP gene to determine if this would be effective in providing resistance to a slowly accumulating low titre virus. In addition they used the CaMV 35S promoter which is known to have a higher activity in phloem cells than in epidermal or mesophyll cells (Jefferson *et al.*, 1987) and could add to an increased resistance to PLRV using the antisense construct. Results conclusively showed that the level and pattern of protection for PLRV were similar in transgenic plants expressing either positive-sense or antisense CP transcripts, independent of virus inoculum. The authors suggested that the resistance produced occurs at an early stage when both positive and negative RNA strands of the virus are at low levels. Another possibility is competition by the expressed antisense RNA for essential viral or host components, such as the replicase.

In the cases where antisense RNA had little or no effect on virus accumulation and spread (Baulcombe *et al.*, 1987), the results may be explained as follows: (*i*) In most cases the extreme 5' and 3' untranslated sequences have been excluded. Including these sequences may prove more effective through their roles in translation and viral RNA replication (Powell *et al.*, 1989). (*ii*) Dilution of the antisense RNA in the cytoplasm would allow the replication of sense viral RNA until an excess was obtained. (*iii*) The effect of encapsidation: the virus enters the plant cell as a complete virion particle; the viral RNA is not presented in its entirety to the cytoplasm, but instead only partially. The 5' end is decapsidated in the case of TMV (Mundry *et al.*, 1991), or released through a hole in the capsid in the case of isometric viruses (Brisco *et al.*, 1986; Adrian *et al.*, 1992). Ribosomes and other factors become associated and the RNA is translated. For structural reasons therefore, it is unlikely that encapsidated RNA will be accessible to antisense inhibition.

1.3 SUMMARY AND CONCLUSIONS

It is clear that CP-mediated resistance can reduce virus infection and disease development for a number of different host:virus systems. The manifestations or phenotypes of CP-mediated resistance have been explored at fundamental levels in a limited number of examples. What is becoming evident is that there are similarities as well as differences in different host:virus combinations. The pattern that appears to be emerging is that the most significant differences are more likely due to differences between the viruses and their modes of infection and replication, rather than due to the host.

Understanding of the mechanism of CP-mediated resistance is increasing. The results summarized here also shed some light on the mechanism of cross protection. As was mentioned it has been suggested that CP-mediated protection is a part of the complex mechanism of cross protection.

In a number of cases, plants expressing the CP of a particular virus are resistant to infection when inoculated with virions, but not when inoculated with the viral RNA of that virus. The present consensus is that the endogenous CP prevents cotranslational disassembly, an early event in the establishment of viral infection. When plants are completely protected against challenge RNA, which is sometimes the case, it is likely that the effectiveness is due to more than the simple presence of higher levels of CP in the leaves prior to challenge

inoculation. Here we see the possible influence of other suggested mechanisms such as RNA:RNA interactions or the blocking of other specific viral or host processes at a later stage in the infection cycle.

A practical and important question in any type of disease resistance relates to the breadth of resistance conferred by the gene. Several types of experiments have suggested that CP-mediated resistance may be relatively broad in activity. It has been documented that this resistance is effective against the virus from which the CP sequence was obtained, and in some cases to related strains with up to 40% sequence dissimilarity. The spectrum of resistance has not been shown to be effective between virus groups. There is no general rule which allows one to predict the degree of sequence similarity between the expressed CP and the challenge viral CP which will result in protection. In the case of AlMV a single amino acid has been shown to be critical for CP-mediated protection.

A component of the resistance response appears to be related to reduced systemic spread or long-distance movement of the virus. Since the CP of many viruses is involved in this later event in the infection cycle, the resistance may be due in part to excess CP interfering with movement of the virus into or out of the phloem. Analysis of cells around the vascular system of inoculated leaves from both CP(+) and non-transgenic plants would provide evidence for or against this hypothesis.

As illustrated in Table 1.1, CP-mediated resistance has been and is still being applied to develop resistance to virus infections in a number of crop plants. Small-scale field trials have demonstrated that this resistance is effective in controlling both mechanically transmitted as well as insect-vectored viruses (Gonsalves *et al.*, 1991; Nelson *et al.*, 1988; Sanders *et al.*, 1992). A better understanding of the cellular and molecular mechanisms may lead to new-generation CP genes that improve resistance beyond the current levels.

CHAPTER 2

Molecular cloning and determination of the nucleotide sequences of the coat protein genes of two plant ssRNA viruses

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CHAPTER 2

Molecular cloning and determination of the nucleotide sequences of the coat protein genes of two plant ssRNA viruses.

SUMMARY

The complete complementary DNA sequences of the cucumber mosaic virus-Wemmershoek (CMV-Wem) CP gene (657 bp), and the tobacco necrosis virus-F5P (TNV-F5P) CP gene (831 bp) were cloned and subjected to sequence analysis. The CMV-Wem isolate has a CP ORF of 654 nucleotides which is probably translated into a polypeptide of 218 amino acids. Multiple nucleotide and amino acid sequence alignments of CMV CP genes from subgroups I and II showed that CMV-Wem belongs to subgroup II. CMV-Wem displayed a striking similarity to CMV-WL (subgroup II), with a 98.6% nucleotide and 98% amino acid sequence identity.

TNV-F5P has a CP ORF of 831 nucleotides which is probably translated into a CP of 276 amino acids. Comparison of nucleotide sequences and amino acid sequences of TNV strains A and D with that of the South African isolate F5P showed that these strains and isolate F5P are related. TNV-F5P CP displayed significant nucleotide sequence similarity (96%) to the corresponding sequence of TNV-A. The similarity is less striking between TNV-F5P and TNV-D with an overall 52.5% nucleotide identity.

2.1 INTRODUCTION

2.1.1 Cucumber mosaic virus genome structure

Cucumber mosaic virus (CMV) is one of the most economically important plant viruses because of its world-wide distribution, extensive host range (over 800 plant species), its effects on yields of agriculturally important crop plants, and the large number of different biological strains isolated (Francki, 1979; Kaper and Waterworth, 1981). More than 60 strains have been reported (Hayakawa *et al.*, 1989), and they appear to fall into two biologically and biochemically distinguishable groups. Subgroup I includes strains C, D, Fny, and Y; subgroup II includes strains Q and WL. Within each subgroup CP amino acid sequences

share more than 95% sequence identity. However there is a lower degree of similarity between the subgroups (approximately 80%; Quemada *et al.*, 1989).

This icosahedral, multicomponent virus belongs to the family Bromoviridae, and is the type member of the genus Cucumovirus (Rybicki, 1993). It has a positive-sense single-stranded RNA genome consisting of three RNA species which are designated RNA 1, 2 and 3 in order of decreasing M_r (Fig 2.1). A subgenomic RNA (RNA 4) is generated during replication and is reported to serve as a monocistronic messenger for the *in vivo* synthesis of the CP (Symons, 1985). The genome of CMV resembles that of other plant tripartite viruses, which have the following properties in common:(a) 5' m^7G cap structure on all four RNAs, (b) a conserved untranslated "t-RNA like" structural sequence at the 3'-terminal ends of all genome segments (Symons, 1979), and (c) the general organization of viral cistrons amongst three distinct RNAs (Rezaian *et al.*, 1984; Matthews, 1991). The RNA 3 genome, which is approximately 2220 nucleotides in length, encodes two translation products, a 3a protein at the 5' end, and the CP (Gould and Symons, 1982). The CP gene yields a protein of 218 amino acids (Owen *et al.*, 1990). The expression of RNAs 1 and 2 results in the formation of a membrane-bound RNA-dependent RNA polymerase essential for the replication of CMV RNA (Nitta *et al.*, 1988a).

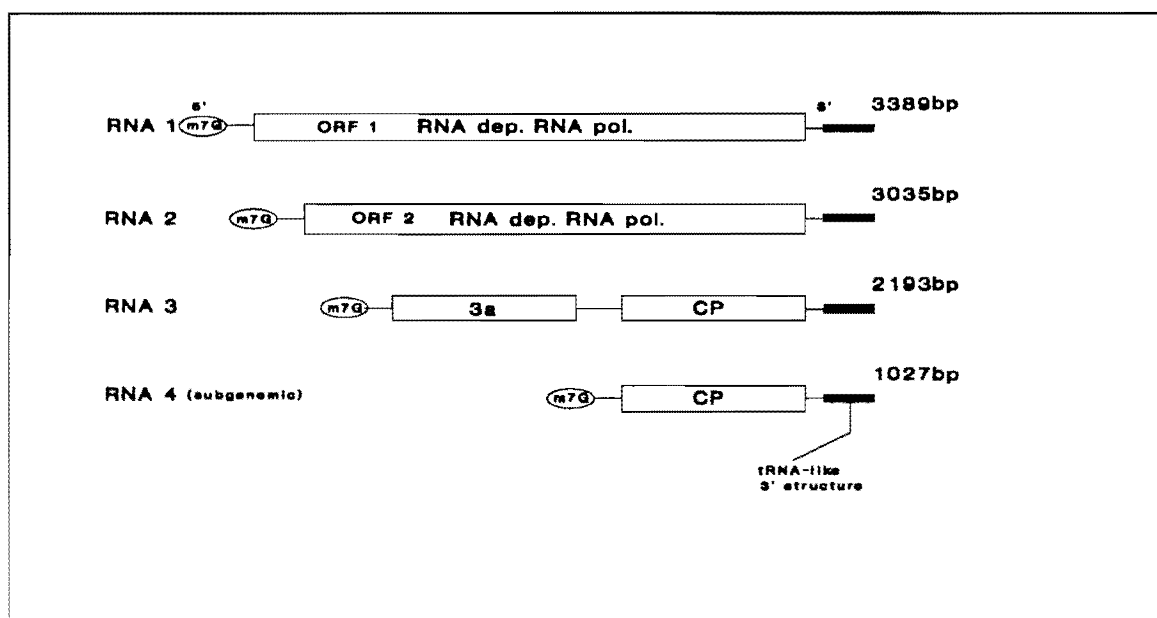

Fig. 2.1 Diagrammatic representation of the cucumber mosaic virus (CMV) RNA genome. Adapted from Symons (1985).

2.1.2 Tobacco necrosis virus genome structure

A new disease of maize was noticed for the first time in the 1988 winter season in the Eastern Transvaal Lowveld, South Africa. The destructiveness of the disease justified a detailed investigation. Initial studies showed that a complex of viruses was present in the natural infections: this included CMV and another virus which was later identified as tobacco necrosis virus (TNV) (von Wechmar *et al.*, 1990).

Necrovirus is a recently coined name for a very small virus group, presently encompassing TNV (A strain) as the type member, and chenopodium necrosis virus. Other possible members are carnation yellow stripe virus and lisianthus necrosis virus. TNV was identified as early as 1935, and although it has a number of unusual features this virus has been studied less extensively and in fewer laboratories than most of the long-known plant viruses (Fraenkel-Conrat, 1988). TNV is a small icosahedral, fungus-transmitted virus, which is often associated with satellite virus (STNV), a smaller particle which is unable to replicate in the absence of TNV (van Emmelo *et al.*, 1987).

The unipartite positive-sense single-stranded RNA genome of TNV is 3.8 - 4.0 kb long and shares less than 2% identity with STNV RNA. It has no cap structure, does not possess a covalently linked virion protein (Vpg), and lacks a 3' poly(A) tail and amino acid-binding capacity (Fraenkel-Conrat, 1988).

Until very recently most attention given to TNV was focused on the molecular biology of a single STNV isolate. Now the almost complete nucleotide sequence of the TNV strain A RNA genome (Meulewaeter *et al.*, 1990), and the complete nucleotide sequence of TNV strain D have been established (Coutts *et al.*, 1991). The nucleotide sequence of TNV RNA reveals that this virus has a genome organization similar to that of carnation mottle Carmovirus (CarMV), turnip crinkle virus (TCV) and cucumber necrosis Tombusvirus (CNV), but resembles the Carmoviruses more closely overall. The genomic organization and translation strategy proposed for TNV-A and TNV-D is illustrated in Fig 2.2

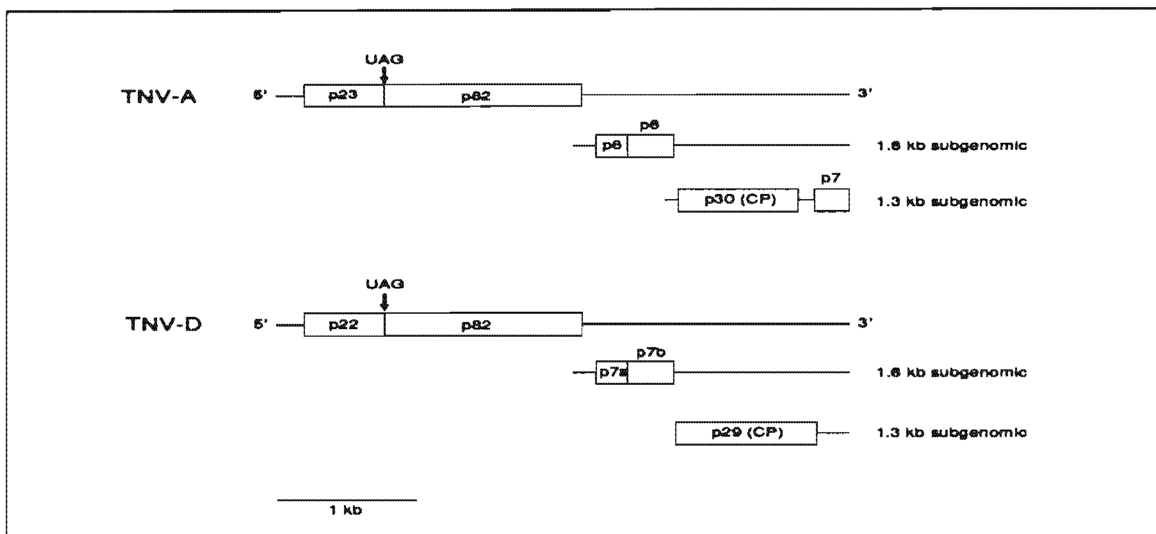

Fig. 2.2 Diagrammatic representation and comparison of the genome organizations of tobacco necrosis virus (TNV) strains A and D. The main ORFs for each virus genome are shown as boxes, with the arrows indicating termination codons which may be read through (Coutts *et al.*, 1991).

The dsRNA profile obtained from early studies of TNV-infected tobacco leaves revealed two subgenomic species of 1.6 kb and 1.3 kb (Condit and Fraenkel-Conrat, 1979). This has been confirmed by Meulewaeter *et al.*, 1990 and Coutts *et al.*, 1991. In the case of TNV-A and TNV-D the larger of the two subgenomic species is the mRNA for ORF3 (function unknown), and the smaller 1.3 kb subgenomic RNA acts as a template for the expression of CP. Both dsRNA subgenomic species have ssRNA counterparts in infected plant extracts (Coutts *et al.*, 1991).

The only known TNV structural gene product is the CP which is encoded by the largest 5'-proximal ORF of the 1.3 kb RNA, designated p30 and p29 for strains A and D respectively (Meulewaeter *et al.*, 1990; Coutts *et al.*, 1991). The sequence of p29 of TNV-D shows striking similarity with p30 of TNV-A (Coutts *et al.*, 1991), and with southern bean mosaic virus (SBMV) CP. The CP subunits of a number of icosahedral viruses are arranged into four distinct domains called R (random, N-terminal), an arm (a) which connects R and S (shell domain), and P (projecting, C-terminal). Both TNV-A and TNV-D coat proteins lack a P domain, along with southern bean mosaic virus (SBMV) (Meulewaeter *et al.*, 1990; Coutts *et al.*, 1991). It was speculated that the P domain region was involved in fungal transmission (Riviere *et al.*, 1989) or in the infection of cucumber. However, because of the lack of a similar sequence in the structural protein of TNV, it seems that this

region is not essential for the transmission by *Olpidium brassicae* (Muelewaeter *et al,*. 1990).

2.1.3 Coat protein-mediated resistance

For an ever increasing number of agronomically important plant viruses it has been shown that transformation of hosts with a translationally active form of the coat protein gene of a given virus may confer resistance to infections and/or disease development by the corresponding virus or related strain. This form of engineered resistance is now generally referred to as `coat protein-mediated resistance.' This has been extensively reviewed in Chapter 1 of this thesis.

The first step in the construction of the CP gene expression system is the isolation of cloned cDNA that encompasses the entire CP ORF. This is relatively straightforward for viruses with CPs that are encoded by subgenomic RNAs, as these ORFs have a favoured CP ATG initiation codon (Beachy *et al.*, 1990). However, this is not the case for viruses in the Potyvirus group (Carrington *et al.*, 1990), for example, or for other viruses whose CPs result from proteolytic cleavage of larger precursors (Matthews, 1991).

This chapter reports the molecular cloning and determination of the nucleotide sequences of a 1 kb fragment of the CMV-Wem RNA 3 genome, encompassing the entire CP gene, and a 1.2 kb 3' terminal fragment of the TNV-F5P genome, also encompassing the entire CP ORF. By characterizing and sequencing these cDNA clones the authenticity of the CMV and TNV CP genes were determined, prior to their subcloning into the appropriate vectors for expression in *E. coli* and tobacco.

2.2 MATERIALS AND METHODS

Methods and techniques not described or referenced here were performed as described by Sambrook *et al.*, 1989.

2.2.1 Virus isolates, propagation and purification

All virus strains and isolates used were kindly donated by Assoc. Prof. M. B. von Wechmar (University of Cape Town collection). CMV-Wem was sap transmitted from *Cucurbita pepo* to *N. tabacum* cvs. Soulouk and Xanthi and *N. benthamiana* to

propagate the virus for purification. Due to the instability of the virion particles all procedures were carried out on ice using ice-cold buffers and equipment. Tobacco leaves (165-200 g) systemically infected with CMV were harvested seven to eight days post inoculation, and homogenised in 600 ml 0.2 M sodium phosphate buffer (pH 7.5) containing 0.1% thioglycollic acid in a Sorvall blender. The homogenate was expressed through a double layer of cheesecloth and centrifuged at 10 000 rpm for 10 min (low speed) in a Sorvall GSA rotor. Triton-X-100, NaCl and polyethylene glycol were added to the supernatant to final concentrations of 2% v/v, 2.5% w/v and 8.5% w/v respectively. After stirring for 15 min at 4°C, the precipitate was collected by low speed centrifugation and resuspended in 60 ml (1/10 original vol) 0.05 M Tris-HCl (pH 8.8), clarified at low speed, and the supernatant ultracentrifuged at 34 000 rpm for 90 min in a Beckman type 35 rotor. The pellet was allowed to resuspend overnight (O/N) in 0.05 M Tris-HCl (pH 8.8). The virus was further purified by centrifugation in a 10-35% (w/v) continuous sucrose gradient in 0.05 M Tris-HCl (pH 8.8) at 27 000 rpm for 150 min in a Beckman SW28 rotor, followed by fractionation on an ISCO density gradient fractionator with UA-5 UV adsorption monitor and 254 nm filter. The virion-containing fractions were diluted 1:4 in 0.05 M Tris-HCl (pH 8.8) and precipitated by ultracentrifugation. The final pellets were resuspended in the same buffer and kept at 4°C.

TNV-F5P infected primary leaves of *N. tabacum* cvs Soulouk and Xanthi showing local lesions were harvested and ground ultra-fine with carborundum using a mortar and pestle. After grinding, 0.01 M phosphate buffer (pH 7) was added in a 1:5 (w/v) ratio. The sap was clarified by centrifugation at 7000-8000 rpm for 10 min in a Sorvall GSA rotor. Triton-X-100 was added to a final concentration of 1% (v/v), and the mixture stirred for 15 min. The precipitate was collected by ultracentrifugation at 34 000 rpm for 120 min in a Beckman type 35 rotor, and resuspended in 5 ml 0.01 M phosphate buffer (pH 7). The suspension was clarified by centrifugation in a bench top centrifuge for 10 min at 4°C. The virus was further purified by sucrose density gradient centrifugation, as described above. Due to the stability of the virion particles, this could be repeated in order to increase the virus purity.

2.2.2 Extraction of single and double stranded RNA

Single stranded RNA was extracted from sucrose gradient-purified virus preparations by a protocol based on the method described by Gallitelli *et al.*

(1985). Preparations of CMV-Wem and TNV-F5P were heated at 60°C for 5 min in the presence of 10 mM Tris-HCl (pH 8.25); 1% (w/v) SDS; 1 mM EDTA. The samples were then extracted with phenol/chloroform and ethanol-precipitated. Pellets were resuspended in sterile double distilled water, and the RNA used for further manipulations, or stored frozen at either -20°C or -70°C. The concentration was estimated by measuring the UV absorption at 260 nm, with an OD of 1 corresponding to a concentration of 25 µg/ml.

Double stranded CMV-Wem and TNV-F5P RNA was isolated directly from either fresh or frozen infected *N. tabacum* leaf material, using methods described by Dodds *et al.* (1984) and Garger and Turper, (1986).

2.2.3 Synthesis and cloning of complementary DNA from single-stranded CMV-Wem and TNV-F5P.

2.2.3.1 cDNA synthesis from single-stranded CMV-Wem RNA and molecular cloning

First and second strand synthesis of CMV-Wem cDNA was carried out using the cDNA Synthesis System Plus (Amersham International, Gubler and Hoffman, 1983). Methylmercury hydroxide treatment (10 mM final concentration, 30 min at room temperature (RT); Payvar and Schimke, 1979) was performed immediately prior to first strand synthesis to allow thorough denaturation of the ssRNA. The reaction was stopped using 1/5 vol 700 mM β-mercaptoethanol. The first strand synthesis reaction was primed using an oligonucleotide (5'-CGTTTAGGGACTTCAGGCAG-3'), complementary to nucleotides 1956 to 1976 of RNA 3 of CMV-Q (Davies and Symons, 1988).

Both first and second strand synthesis reactions were assayed by the incorporation of [α-^{32}P]dCTP. The unincorporated [α-^{32}P]dCTP was separated from the labelled cDNA molecules using Sephadex-G50 (Pharmarcia) pasteur column chromatography. The radioactive fractions in the leading peak were pooled and the cDNA was ethanol precipitated using the ethanol procedure (4% (v/v) 4 M ammonium acetate and 2 vols EtOH). The pellet was resuspended in 10 µl TE buffer, pH 7.6, and the sample applied to a Sepharose CL-4B (Pharmarcia) column equilibrated with TE buffer. The column was eluted using TE buffer and twenty 50 µl fractions collected. Fractions 9-20 were counted on a Packard Tri-Carb Model 460 scintillation counter using a water-based

scintillation cocktail (Scintillator 299™, Packard). The fractions from three distinct peaks were pooled and again EtOH precipitated. The blunt-ended ds cDNA was resuspended in 5 µl sterile TE buffer. This DNA was cloned into the *Sma*I site of the pUC19 polylinker (Rutledge *et al.*, 1988; Yanisch-Perron *et al.*, 1985)

Recombinant plasmids were used to transform *E. coli* LK111 competent cells (Chung and Miller, 1988). White colonies, indicative of insertional inactivation of the β-galactosidase gene of the vector (Vieira and Messing, 1982), were selected for colony hybridization assays (Buluwela *et al.*, 1989). These were probed with a [γ-^{32}P]dATP 5'-end labelled CMV dsRNA 3 probe. DsRNA 3 was gel purified by the "freeze-squeeze" method (Tautz and Renz, 1983). Clones which hybridized to the probe were analyzed by restriction enzyme digestion and gel electrophoresis to determine the exact size of the inserts. Putative positive recombinants were also screened using the following procedure to isolate total bacterial DNA. Cells from transformed colonies were transferred to micro centrifuge tubes containing 750 µl LB medium supplemented with 100 µg/ml ampicillin. After shaking vigorously at 37°C for 2-3 h, cells were harvested by centrifugation and resuspended in 40 µl STE buffer. An equal volume of phenol:chloroform:isoamylalcohol (25:24:1) was added, the solution thoroughly vortexed, and the phases separated by centrifugation. Approximately 5 µl of the aqueous phase was loaded directly onto a 0.8% agarose gel. Clones which seemed to be of suitable size were further analysed by restriction enzyme digestion and gel electrophoresis.

2.2.3.2 cDNA synthesis from single-stranded TNV-F5P and molecular cloning

Two separate reactions were performed to synthesize TNV cDNA, with and without the addition of [α-^{32}P]dCTP. First and second strand reactions were carried out using the cDNA Synthesis System Plus (Amersham International) (see above). The first strand synthesis reaction was primed with an oligonucleotide (5'-CTAGACGTTCATTGTTGGGTTG-3') complementary to nucleotides 3422 to 3443 of the TNV-A RNA genome (Meulewaeter *et al.*, 1990). The addition of [α-^{32}P]dCTP was in order to monitor the efficiency of the reaction. Blunt-ended ds cDNA was EtOH precipitated and resuspended in 5 µl TE buffer pH 7.5.

Before cloning, the entire ds cDNA sample was electrophoresed on a 0.8% TAE agarose gel. Gel slices were excised corresponding to the expected size of the CP

gene and larger, and the DNA purified from the agarose using Geneclean™ (BIO101, USA; Vogelstein and Gillespie, 1979). The purified cDNA was ligated into the *EcoRV* site of the pBluescript SK+ vector polylinker (Stratagene, La Jolla, CA). Recombinant plasmid DNA was transformed into *E. coli* LK111, and white colonies were selected, as described above. Plasmid DNA was isolated from these white colonies and probed with a [γ-^{32}P]dATP 5' end-labelled TNV-specific oligonucleotide probe by dot-blot hybridization. Clones which hybridized to the probe were analysed by restriction enzyme digestion and gel electrophoresis to determine the size of the inserts.

2.2.4 Hybridization analysis of recombinant clones

DNA for probing was radiolabelled with [α-^{32}P]dCTP by nick translation, using the Amersham kit (N.5000, Amersham, UK), as described by the distributors. 5'-End-labelling of dsRNA was performed by a modification of the method described by Rosner *et al.*, 1983. DsRNA (1 μg) was resuspended in 10 μl hydrolysis buffer (50 mM Tris-HCl pH 9.5; 1 mM EDTA), and incubated at 88°C for 45 min. Labelling was performed at 37°C for 1 h by the addition of 25 μCi [γ-^{32}P]dATP, 10 U polynucleotide kinase (PNK), 5 μl RNA labelling buffer (50 mM Tris-HCl pH 9.5; 45 mM $MgCl_2$; 5 mM dithiotreitol), in a total volume of 20 μl. The reaction was stopped by the addition of 20 μl 0.5 M EDTA and the unincorporated nucleotides removed by Sephadex G50 pasteur column chromatography. Labelling of ssDNA oligonucleotides was performed as described (Ausubel *et al.*, 1987).

2.2.5 Nucleotide sequence determination

Nucleotide sequencing was performed by a modification of the dideoxy chain termination method of Sanger *et al.* (1977) for dsDNA templates using the Sequenase Kit version 2.0 (U.S. Biochemicals). The primer 5'-CAGGAAACAGCTATGAC-3' (Amersham) was used to sequence the "reverse" strand while 5'-GTTTTCCCAGTCACGAC-3' (US Biochemicals) was used as the "forward" sequencing primer. Labelling and termination reactions were carried out in the presence of 10% DMSO (Winship, 1989).

A recombinant plasmid with a CMV-derived insert, pCDCR379, was selected for sequence analysis. This plasmid was not subjected to unidirectional exonulease III and S1 nuclease shortening, but overlapping deletion mutants were created

using certain unique restriction enzyme sites. The strategy used for sequencing the above insert is shown in Fig. 2.3.

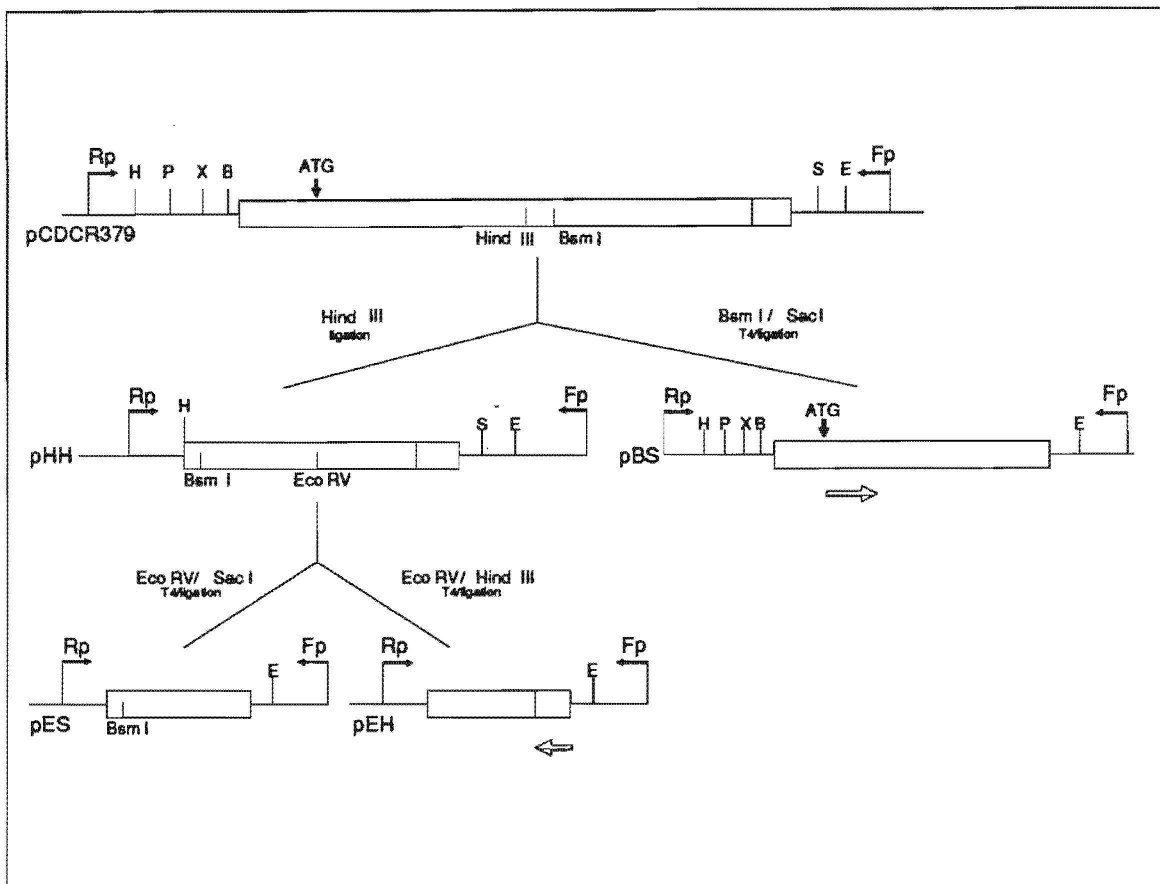

Fig. 2.3	Construction of deletion mutants for sequencing the CMV CP gene region in plasmid pCDCR379. The 474 bp *Hind*III fragment was deleted from pCDCR379 to yield pHH. The plasmid, pCDCR379, was cut with *Bsm*I and *Sac*I, blunt-ended with T4 DNA polymerase, and the vector fragment gel purified with the Geneclean[R] kit (BIO101, USA) and ligated to yield pBS. Similarly pHH was cut with *Eco*RV and then with *Sac*I or *Hind*III, blunt-ended, and the vector fragments ligated to give plasmids pES and pEH respectively. Only selected restriction sites are indicated and plasmid maps are not drawn to scale. Restriction sites: B, *Bam*HI; Bs, *Bsm*I; E, *Eco*RI; H, *Hind*III; P, *Pst*I; S, *Sac*I; X, *Xba*I. Open arrows represent additional sequencing primers. Rp: pUC19 (M13mp19) reverse primer; Fp: universal forward primer (M13mp19).

Two additional primers were used to complete the sequencing. The first was the primer used to direct the cDNA synthesis described in section 2.2.3, and the second was the oligonucleotide 5'-CCTATGGACAAATCTGGATC-3', which has the same sequence as nucleotides 1217 to 1237 of RNA 3 of CMV-Q (Davies and Symons, 1988).

Compilation and analysis of partial genomic sequences was done using programmes from the Genetics Computer Group (GCG, University of Wisconsin) package version 6.1 (Devereux *et al.*, 1984) run on a DEC/VAX 6000-330 mainframe computer. Coat protein sequences from the following isolates were used for comparisons: CMV-Wem (this work), CMV-C (Quemada *et al.*, 1989), CMV-Chi (Chinese isolate, Hu *et al.*, 1989), CMV-Fc (Shintaku, 1991), CMV-Fny (Owen *et al.*, 1990), CMV-I17F (Noel and Ben Tahar, 1989), CMV-Kin (GenEMBL, unpublished), CMV-O (Hayakawa *et al.*, 1989), CMV-P6 (Shintaku, 1991), CMV-PR1, CMV-PR36 and CMV-PR50 (GenEMBL, unpublished), CMV-WL (Quemada *et al.*, 1989), CMV-Y (Nitta *et al.*, 1988b) and CMV-Q (Davies and Symons, 1988)

A recombinant plasmid with a TNV derived insert, pSTN4, was selected for sequence analysis. This plasmid was subjected to unidirectional shortening (Henikoff, 1984) from each end of the insert, using exonuclease III and S1 nuclease (Boehringer Mannheim). This produced sets of overlapping deletion mutants for sequencing. Compilation and analysis of sequences was carried out as described above.

2.3 RESULTS AND DISCUSSION

2.3.1 Virus propagation and purification

N. benthamiana was found to be an excellent host for CMV-Wem purification. CMV, well known to be very unstable, retained its stability and infectivity for up to three weeks when purified from this host (R. Brand, personal communication). However, large yields could also be purified from *N. tabacum* cvs. Xanthi and Soulouk. Sucrose gradient fractionation often gave the first clear indication of the presence of one or more contaminating viruses.

Nicotiana tabacum cvs. Soulouk and Xanthi were found to give sufficiently good yields of TNV particles. Due to the stability of the virion particles sucrose gradient fractionation could be performed several times depending on the intended purity of the virus. TNV particles formed a unique tight and discrete band in the lower half of the 10-35% sucrose density gradient tube. This made it easy to distinguish this virus from possible contaminating viruses.

2.3.2 Extraction of single and double-stranded RNA

Double stranded CMV-Wem RNA extracted from infected *N. benthamiana, N. tabacum* cvs. Soulouk and Xanthi, as well as ssRNA extracted from the purified virus, showed the characteristic 4-band pattern for CMV. RNA sizes correspond well with sizes estimated for other strains of CMV (Francki *et al.*, 1985) (Fig. 2.4A).

An approximately 4 kb ssRNA was isolated from purified TNV propagated in and isolated from *N. tabacum* cvs. Soulouk and Xanthi. This is the typical size of TNV RNA as described by Condit and Fraenkel-Conrat (1979) (Fig. 2.4A).

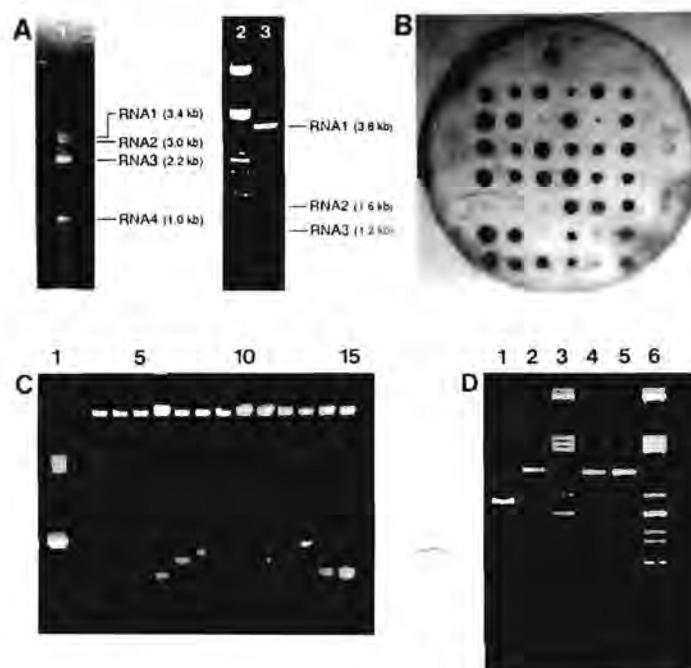

Fig. 2.4 [A] Agarose gel electrophoresis of dsRNA of CMV-Wem (lane 1) and TNV-F5P (lane 3). Lane 2, λ DNA (*Pst*I). [B] Colony hybridization using [γ-^{32}P]dATP end-labelled CMV-Wem dsRNA 3 as a probe to identify *E. coli* LK111 colonies containing recombinant plasmids with CMV-derived cDNA inserts. Positive control (+), CMV dsRNA. Negative control (-), pUC19. [C] Gel electrophoresis of CMV-derived cDNA cloned into the *Sma*I site of pUC19. Total bacterial DNA was extracted from *E. coli* LK111 by the rapid mini-preparation procedure. Lane 1, pUC19 carrying a 900 bp insert (size marker). Lane 2, pUC19. Lanes 3-15, CMV-derived cDNA recombinants. [D] Restriction enzyme analysis of pCDCR379 and pCDCR359. Lane 1, pUC19 (*Sma*I). Lane 2, pCDCR379 (*Bam*HI). Lane 3 & 6, λ DNA (*Pst*I). Lane 4 & 5, pCDCR359 (*Bam*HI).

2.3.3. Synthesis and cloning of complementary DNA

Low yields were obtained of double stranded cDNA synthesized *in vitro* from CMV-Wem dsRNA templates. The poor efficiency was attributed to the first strand synthesis reaction as the second strand reaction produces an efficiency of higher than 90%. Transformation of competent *E. coli* LK111 with 2 µl of the ligation mixture consisting of 20 ng insert and vector DNA in an equimolar ratio, yielded white colonies on indicator plates. Forty-two were selected for colony hybridization assays, of which 34 hybridized to the [γ-^{32}P]dATP 5' end-labelled CMV dsRNA 3 probe (Fig. 2.4B). Another 80 white colonies were screened using a rapid total bacterial DNA isolation and plasmid sizing procedure (Fig. 2.4C).

Two recombinant plasmids containing CMV sequences, pCDCR359 and pCDCR379, were chosen for further characterization. They carried the largest inserts of those screened by plasmid size and also hybridized positively with the CMV RNA 3 specific primer (results not shown). This was confirmed when total CMV RNA and the individual CMV RNAs were probed with pCDCR359 and pCDCR379 [α-^{32}P]dCTP nick translated probes, respectively (results not shown). Restriction enzyme analysis of pCDCR359 and pCDCR379 established that both inserts were larger than 800 bp, and that the pCDCR379 had an insert slightly larger than that of pCDCR359 (Fig. 2.4D). An insert size larger than 755 bp, from the cDNA priming site, would encompass the entire CMV CP gene ORF (Davies and Symons, 1988). Sequence from the "reverse" primer end of the pCDCR379 clone confirmed that the entire CMV CP ORF had been transcribed into cDNA and cloned.

In the case of the cDNA synthesised from TNV-F5P RNA, the incorporation of [α-^{32}P]dCTP nucleotides gave a clear indication that the first strand synthesis reaction was priming from the DNA primer complementary to the 3' end of the TNV CP gene. Agarose gel electrophoresis, in which the cDNA was sized against a lambda DNA marker, served as an effective alternative to using column chromatography. Transformation of competent *E. coli* LK111 cells with a ligation mixture consisting of gel purified TNV-F5P cDNA and *Eco*RV linearised Bluescript pSK$^+$ yielded 150 white colonies on indicator plates. The plasmids from 22 of these were screened, and it was evident that the majority fell into the predicted size range. Approximately 90% of the recombinant plasmids had inserts the same size or larger than the CMV CP gene insert in Bluescript pSK$^+$ (Fig. 2.5A). One clone hybridized positively when probed with a [γ-^{32}P]-dATP

end-labelled ssDNA oligonucleotide TNV RNA specific primer (Fig. 2.5B). Recombinant plasmid pSTNB5 had an insert of about the correct size to encompass the entire TNV CP ORF, and showed the strongest hybridization to the probe. It also hybridized to TNV ssRNA and dsRNA (Fig. 2.5B).

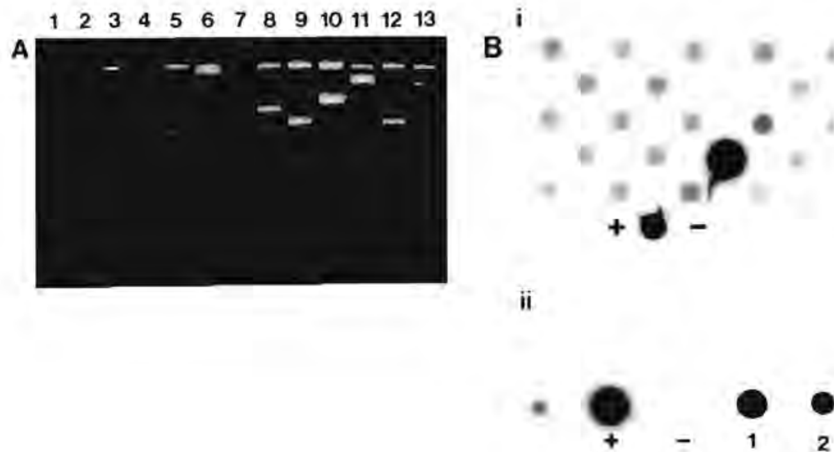

Fig. 2.5	[A] Gel electrophoresis of total bacterial DNA from TNV-derived cDNA cloned into the *Eco*RV site of Bluescript pSK+. Lanes 1 & 7, uncut pCDCR379 (1 kb size marker). Lanes 2-6 and 8-13, TNV-derived cDNA recombinants. [B](i) Dot-blot hybridization to identify TNV-derived cDNA clones using a [γ-^{32}P]dATP end-labelled TNV RNA specific oligonucleotide primer probe. Positive control (+), TNV ssRNA. Negative control (-), pSK. (ii) TNV ssRNA (1) and dsRNA (2) probed with [α-^{32}P]dCTP-labelled pSTNB5. Negative control (-), BMV dsRNA. Positive control (+), pSTNB5.

Preliminary sequence data of both the 3' and 5' ends were compared to the published data (Meulewaeter *et al.*, 1990), which confirmed that the cloned fragment encompassed the entire TNV CP gene.

2.3.4 Nucleotide sequence determination and analysis

2.3.4.1. CMV-Wem cDNA sequence

Four deletion mutants were constructed using unique restriction enzyme sites in the insert and the vector polylinker region (Fig. 2.3). Preliminary 3'-terminal sequencing of the CMV-Wem cDNA insert indicated nucleotide sequence similarity with CMV Q (Davies and Symons, 1988) of the order of 96%. It was therefore assumed that the pCDCR379 CMV-Wem cDNA restriction enzyme map would be almost identical to that of CMV Q; thus the CMV Q map was used

to generate the deletion mutant strategy. The advantage of this strategy over exonuclease III/S1 shortening is that both DNA strands of the deletion mutant can be sequenced to completion. The nucleotide sequence of the 755 bp CMV CP cDNA clone is shown in Fig. 2.6.

Since the first reports on the nucleotide sequences of the multicomponent genome of CMV strain Q (Gould and Symons, 1982; Rezaian *et al.*, 1984), numerous reports on the sequence analysis of the CP region of other CMV strains and isolates have been published. It was, and still is of great interest to discover whether or not differences in nucleotide sequences relate to each strain's characteristics, such as symptoms of the disease or host specificity. Recently the chlorosis-inducing determinant of CMV-M RNA 3 has been mapped by recombination between restriction endonuclease fragments of cDNA clones of CMV-Fny and CMV-M RNA 3 (Shintaku, 1991). The author also compared the CP genes of these chlorosis-inducing strains of CMV at the amino acid level with those of two other chlorosis-inducing strains and four mosaic-inducing strains. A proline was found to be present at amino acid 129 of all the mosaic-inducing strains, whereas that position was occupied by either a serine or a leucine in the CPs of all the chlorosis-inducing strains. It is likely that chlorosis induction is a function of structural alteration in the CP caused by the change in amino acid at position 129. Whether the CP alone, CP and viral RNA, or virions derived from such CP subunits are responsible for the induction of chlorosis, remains to be determined.

Using the PRETTY option of the GCG programme package, and the separate CLUSTAL programme (Higgins *et al.*, 1992) the CP gene nucleotide sequences of 15 CMV strains and isolates were aligned, a CP consensus sequence deduced (Fig. 2.7) and a matrix of percent pairwise sequence dissimilarities calculated (Table 2.1). All CP sequences given in Fig. 2.7 contained 657 residues, including the 3 stop codon nucleotides, giving polypeptides of 218 amino acids. The similarities of the nucleotide and amino acid sequences of the CP gene of CMV-Wem were greatest to those of CMV-WL in subgroup II (98.6% and 97%, respectively), and least to CMV-Y in subgroup I (76.5% and 82%, respectively). These values are consistent with those described in the literature (Quemada *et al.*, 1989): within the subgroups, the sequences are highly conserved with greater than 95% nucleotide sequence identity between any two strains. However, these figures are in the region of 78% for nucleotide sequences of two strains in different subgroups.

```
CCT ATG GAC AAA TCT GAA TCT CCC AAT GCT AGT AGA ACC TCC CGG    45
    Met Asp Lys Ser Glu Ser Pro Asn Ala Ser Arg Thr Ser Arg    14
    [CP⇒
CGT CGT CGC CCG CGT AGA GGT TCT CGG TCC GCT TCT GGT GCG GAT    90
Arg Arg Arg Pro Arg Arg Gly Ser Arg Ser Ala Ser Gly Ala Asp    29

GCA GGG TTG CGT GCT TTG ACT CAG CAG ATG CTG AAA CTC AAT AAA   135
Ala Gly Leu Arg Ala Leu Thr Gln Gln Met Leu Lys Leu Asn Lys    44

ACC CTC GCC ATT GGT CGT CCC ACT CTT AAC CAC CCA ACC TTC GTG   180
Thr Leu Ala Ile Gly Arg Pro Thr Leu Asn His Pro Thr Phe Val    59

GGT AGT GAA AGC TGT AAA CCC GGT TAC ACT TTC ACA TCT ATT ACC   225
Gly Ser Glu Ser Cys Lys Pro Gly Tyr Thr Phe Thr Ser Ile Thr    74

CTG AAA CCG CCT GAA ATT GAG AAA GGT TCA TAT TTC GGT AGA AGG   270
Leu Lys Pro Pro Glu Ile Glu Lys Gly Ser Tyr Phe Gly Arg Arg    89

TTG TCT TTG CCA GAT TCA GTC ACA GAC TAT GAT AAG AAG CTT GTT   315
Leu Ser Leu Pro Asp Ser Val Thr Asp Tyr Asp Lys Lys Leu Val   104

TCG CGC ATT CAA ATC AGG ATT AAT CCC TTG CCG AAA TTT GAT TCT   360
Ser Arg Ile Gln Ile Arg Ile Asn Pro Leu Pro Lys Phe Asp Ser   119

ACC GTG TGG GTT ACA GTT CGG AAA GTA CCT TCA TCA TCC GAT CTT   405
Thr Val Trp Val Thr Val Arg Lys Val Pro Ser Ser Ser Asp Leu   134

TCC GTC GCC ACC ATC TCT GCT ATG TTT GGC GAT GGT AAT TCA CCG   450
Ser Val Ala Thr Ile Ser Ala Met Phe Gly Asp Gly Asn Ser Pro   149

GTT TTG GTT TAT CAG TAT GCT GCG TCC GGA GTT CAG GCC AAC AAT   495
Val Leu Val Tyr Gln Tyr Ala Ala Ser Gly Val Gln Ala Asn Asn   164

AAG TTA CTT TAT GAC CTG TCC GAG ATG CGT GCT GAT ATC GGC GAC   540
Lys Leu Leu Tyr Asp Leu Ser Glu Met Arg Ala Asp Ile Gly Asp   179

ATG CGT AAG TAC GCC GTC CTG GTT TAC TCG AAA GAC GAT AAA CTA   585
Met Arg Lys Tyr Ala Val Leu Val Tyr Ser Lys Asp Asp Lys Leu   194

GAG GAG GAC GAG ATT GTA CTT CAT GTC GAC GTC GAG CAT CAA CGA   630
Glu Glu Asp Glu Ile Val Leu His Val Asp Val Glu His Gln Arg   209

ATT CCT ATC TCA CGG ATG CTC CCG ACT TAG TCC GTG TGT TTA CCG   675
Ile Pro Ile Ser Arg Met Leu Pro Thr ***                       217

GCG TCC GAA GAC GTT AAA CTA CAC TCT CAA TCG CGA GTG TTG AGT   720


TGG TAG TAT TGC TCC AAA CTG CCT GAA GTC CTA AAC G             757
```

Fig. 2.6 Nucleotide sequence of the CMV-Wem coat protein region. The predicted amino acid sequence is shown under the nucleotide sequence. Asterisks (***) indicate the stop codon. The primer binding site for first-strand cDNA synthesis is underlined in the 3'-untranslated region.

```
           1                                                    50                                                  100
Consensus  ATGGACAAAT CTGAATCAAC CAGTGCTGGT CGTAACCGTC GACGTCGTCC GCGTCGTGGT TCCCGCTCCG CCTCCTCCTC CGCGGATGCT AACTTTAGAG

CMV-Wem    ATGGACAAAT CTGAATCTCC CAATGCTAGT AGAACCTCCC GGCGTCGTCG CCCGCGTAGA GGTTCTCGGT CCGCTTCTGG TGCGGATGCA GGGTTGCGTG
CMV-Q      ---------- ---g---tc- --a----a-- a-a-c-tcc- -g-------g c-cg---a-a ggttctcggt --g-t--tgg t--------a ggg--gc-t-
CMV-WL     ---------- ---g---tc- --a----a-- a-a-c-tcc- -g-------g c-cg---a-a ggttctcggt --g-t--tgg t--------a ggg--gc-t-
CMV-Kin    ---------- ---g---tc- --a----a-- a-a-c-tcc- -g-------g c-cg---a-a ggttctcggt --g-t--tgg t--------a ggg--gc-t-
CMV-C      ---------- ---------- ---------- -------a-- ---------- ---------- ---------- --c------- ---------- ----------
CMV-O      ---------- ---------- ---------- --c------- ---------- ---------- ---------- --c------- ---------- ----------
CMV-Y      ---------- ---------- ---------- ---------- --------tt ---------- ---------- ---------- -t-------- ----------
CMV-Fny    ---------- ---------- ---------- ---------- ---------- ---------- ---------- --c------- ---------- ----------
CMV-Chi    ---------- ---------- ---------- --c------- ---------- ----gc---- -g-g---g-- -t-------- --------gc ----------
CMV-Fc     ---------- ---------- ---------- ---------- ---------- ---------- ---------- -tc------- ---------- ----------
CMV-I17F   ---------- ---------- ---------- ---------- ---------- ---------- ---------- --c------- ---------- ----------
CMV-P6     ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
CMV-PR1    ---------- ---------- ---------- ---------- -g-------- ---------- ---------- ---------- ---------- --t-------
CMV-PR36   ---------- ---------- ---------- ---------- -g-------- ---------- ---------- ---------- ---------- --t-------
CMV-PR50   ---------- ---------- ---------- ---------- -g-------- ---------- ---------- ---------- ---------- ----------


         101                                                   150                                                  200
Consensus  TCTTGTCGCA GCAGCTTTCG CGACTTAATA AGACGTTAGC AGCTGGTCGT CCAACTATTA ACCACCCAAC CTTTGTAGGG AGTGAACGCT GTAAACCTGG

CMV-Wem    CTTTGACTCA GCAGATGCTG AAACTCAATA AAACCCTCGC CATTGGTCGT CCCACTCTTA ACCACCCAAC CTTCGTGGGT AGTGAAAGCT GTAAACCCGG
CMV-Q      ct---a-t-- ----a-gct- a----c---- -a--cc-c-- cat------- --c---c--- ---------- ---c--g--t ------a--- -------c--
CMV-WL     ct---a-t-- ----a-gct- aa---c---- ga--cc-c-- cat------- --c---c--- ---------- ---c--g--t ------a--- -------c--
CMV-Kin    ct---a-t-- ----a-gct- aa---c---- -a--cc-c-- cat------- --c---c--- ---------- ---c--g--t ------a-t- -------c--
CMV-C      ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---g------
CMV-O      ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
CMV-Y      ---------- ---------- --------c- ---------- ---------- ---------- ---------- ---------- ---------- ----------
CMV-Fny    ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---g------
CMV-Chi    ---------- ---a------ ---------- -------g-- ---------- --t-gc---- ---------- ---------- ---------- ----------
CMV-Fc     ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---g------
CMV-I17F   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---g------
CMV-P6     ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---g------
CMV-PR1    ---------g a--------- --------c- ---------- ---------- ---------- ---------- ---------- ---------- ----------
CMV-PR36   ---------- ---------- --------c- ---------- ---------- ---------- ---------- ---------- ---------- -g--------
CMV-PR50   ---------- ---------- --------c- ---------- ---------- ---------- ---------- ---------- ---------- ----------


         201                                                   250                                                  300
Consensus  GTACACGTTC ACATCTATTA CCCTAAAGCC ACCAAAAATA GACCGTGGGT CTTATTACGG TAAAAGGTTG TTATTACCTG ATTCAGTCAC GGAATATGAT

CMV-Wem    TTACACTTTC ACATCTATTA CCCTGAAACC GCCTGAAATT GAGAAAGGTT CATATTTCGG TAGAAGGTTG TCTTTGCCAG ATTCAGTCAC AGACTATGAT
CMV-Q      t-----t--- ---------- ----g--a-- g--tg----t --gaaa--t- -a----tt-- --g------- -ct--g--a- ---------- ---c------
CMV-WL     t-----t--- ---------- ----g--a-- g--tg----t --gaaa--t- -a----tt-- --g------- -ct--g--a- ---------- ---c------
CMV-Kin    t-----t--- ---------- ----g--a-- g--tg----t --gaaa--t- -a----tt-- --g------- -ct--g--a- ---------- a--c------
CMV-C      ---------- ---------- ---------- ---------- -------a-- ---------- ---------- ---c------ ---------- ----------
CMV-O      ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
CMV-Y      ---------- ---------- ---------- ---------- ---------- -------t-- ---------- ---------- ---------- ----------
CMV-Fny    ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---c------ ---------- ----------
CMV-Chi    ---------- --------c- -g--g----- ---g------ ----cc---- -------t-- ---------- ---------- ---------- -----tc---
CMV-Fc     ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
CMV-I17F   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---c------ ---------- ----------
CMV-P6     ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---c------ ---------- ----------
CMV-PR1    ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
CMV-PR36   ---------- ---------- ----t----- ---------- ---------- -------t-- ---------- ---------- ---------- ----------
CMV-PR50   ---------- ---------- ---------- g--------- ---------- ---------- ---------- ---------- ---------- ----------
```

```
           301                                                350                                                400
Consensus  AAGAAGCTTG TTTCGCGCAT TCAAATTCGA GTTAATCCTT TGCCGAAATT TGATTCTACC GTGTGGGTGA CAGTCCGTAA AGTTCCTGCC TCCTCGGACT

CMV-Wem    AAGAAGCTTG TTTCGCGCAT TCAAATCAGG ATTAATCCCT TGCCGAAATT TGATTCTACC GTGTGGGTTA CAGTTCGGAA AGTACCTTCA TCATCCGATC
CMV-Q      ---------- ---------- ------ca-g a--------- ---------- ---------- --------t- ----t--g-- ---g---t-a --a--c--tc
CMV-WL     ---------- ---------- ------ca-g ---------- ---------- ---------- --------t- ----t--g-- ---a---t-a --a--c--tc
CMV-Kin    ---------- ---------- ------ca-g a--------- ---------- ---------- --------t- ----t--g-- ---a---t-a --a--c--tc
CMV-C      ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
CMV-O      -----a---- ---------- ---------- ---------- ---------- c--------- ---------- ---------- ---------- ----------
CMV-Y      ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----t----- ----------
CMV-Fny    ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
CMV-Chi    ---------- ---------- ---------- ---------- ---------- ---------g ---------- ---------- ---------- ----------
CMV-Fc     ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----t----- ----------
CMV-I17F   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
CMV-P6     ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- -----t---- ----------
CMV-PR1    ---------- ---------- ---------- ---------- ---------- ---------- --------a- ---------- ---c------ ----------
CMV-PR36   ---------- ---------- ---------- ---------- ---------- ---------- --------a- ----t----- ---------- ----------
CMV-PR50   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----t----- ---c------ ----------

           401                                                450                                                500
Consensus  TATCCGTTGC CGCCATCTCT GCTATGTTCG CGGACGGAGC CTCACCGGTA CTGGTTTATC AGTATGCTGC ATCTGGAGTC CAAGCCAACA ACAAATTGTT

CMV-Wem    TTTCCGTCGC CACCATCTCT GCTATGTTTG GCGATGGTAA TTCACCGGTT TTGGTTTATC AGTATGCTGC GTCCGGAGTT CAGGCCAACA ATAAGTTACT
CMV-Q      -t-----c-- ---------- --------t- gc--t--taa ---------t t--------- ---------- g--c-----t --g------- -t--g--ac-
CMV-WL     -t-----c-- ---------- --------t- gc--t--taa t--------t t--------- ---------- g--c-----t --g------- -t--g--ac-
CMV-Kin    -t-----c-- ---------- --------t- gc--t--taa t--------t ---------- -------c-- ---------- ---------- -----c----
CMV-C      ---------- ---------- ---------- ---------- ---------- ---------- ----c--c-- --t------- -----t---- ----------
CMV-O      ---------- ---------- --------t- ---------- ---------- ---------- ---------- ---------- -----t---- ----------
CMV-Y      ---------- ---------- ---------- ---------- ---------- ---------- -------c-- ---------- ---------- -----c----
CMV-Fny    ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---c------ ---------- -t--g-----
CMV-Chi    -------ca- ---------- --------tt ---------- ---------- ---------- -------c-- ---------- -----a---- ----------
CMV-Fc     ---------- ---------- ---------- ---------- ---------- ---------- -------c-- ---------- ---------- -----c----
CMV-I17F   ---------- ---------- ---------- ---------- ---------- t--------- ---------- g--c-----t --g------- -t--g--ac-
CMV-P6     ---------- ---------- ---------- ---------- ---------- ---------- -------c-- ---------- ---------- -----c----
CMV-PR1    ---------- ---------- --------t- ---------- ---------- ---------- ---------- ---------- -----t---- ----------
CMV-PR36   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- -----t---- ----------
CMV-PR50   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- -----t---- ----------

           501                                                550                                                600
Consensus  GTATGATCTT TCGGCGATGC GCGCTGATAT AGGTGACATG AGAAAGTACG CCGTCCTCGT GTATTCAAAA GACGATGCGC TCGAGACGGA CGAGCTAGTA

CMV-Wem    TTATGACCTG TCCGAGATGC GTGCTGATAT CGGCGACATG CGTAAGTACG CCGTCCTGGT TTACTCGAAA GACGATAAAC TAGAGGAGGA CGAGATTGTA
CMV-Q      t-----c--g --c-a----- -t-------- c--c------ c-t------- -------g-- t--c--g--- ------aaa- -a----a--- ----a-t---
CMV-WL     t-----c--g --c-a----- -t-------- c--c------ c-t------- -------g-- t--c--g--- ------aaa- -a----a--- ----a-t-c-
CMV-Kin    t-----c--g --c-a----- -t-------- c--c------ c-t------- -------g-- t--c--g--- ------aat- -a----a--- ----a-t---
CMV-C      --t------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
CMV-O      ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
CMV-Y      ---------- ---------- ---------- ---c------ ---------- ---------- ---------- ---------- ---------- ----t-----
CMV-Fny    ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
CMV-Chi    ---------- ---------- ---------- t--c------ c--------- ----a----- ---------- -------tc- ---------- t---------
CMV-Fc     ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
CMV-I17F   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- -a-------- ----------
CMV-P6     ---------- ---------- ---------- ---------- ---------- --a------- ---------- ---------- ---------- t---------
CMV-PR1    ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- -------a-- ----t-----
CMV-PR36   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- -------a-- ----------
CMV-PR50   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----t-----
```

```
              601                                                    650
Consensus     CTTCATGTTG ACATCGAGCA CCAACGCATT CCCACATCTG GGGTGCTCCC AGTCTGA

CMV-Wem       CTTCATGTCG ACGTCGAGCA TCAACGAATT CCTATCTCAC GGATGCTCCC GACTTAG
CMV-Q         --------c- --g------- t-----a--- --t-tc--ac --a------- gact-ag
CMV-WL        --------c- --g------- t-----a--- --t-tc--ac --a------- gact-ag
CMV-Kin       --------c- --g------- t-----a--- --t-tc--ac --a------- gact-ag
CMV-C         ---------- ---------- ---------- ---------- -a-------- -------
CMV-O         ---------- ---------- ---------- ---------a -a--a----- -------
CMV-Y         ---------- --g------- ---------- ---------- -a-------- -------
CMV-Fny       ---------- ---------- ---------- ---------- -a-------- -------
CMV-Chi       --------c- ----a----- ---------- ---------- ---------- ---t---
CMV-Fc        ---------- ---------- ------t--- ---------- -a-------- -------
CMV-I17F      ---------- ---------- ---------- -----g---- -a-------- ----...
CMV-P6        ---------- ---------- ---------- ---------- ---------- -------
CMV-PR1       ---------- ---------- ---------- -----g---- ---------- ---t---
CMV-PR36      ---------- ---------- ---------- -----g---- -a-------- -------
CMV-PR50      ---------- ---------- ---------- -----g---- ---------- -------
```

Fig. 2.7 Alignment of the CMV-Wem coat protein nucleotide sequence with 14 other CMV strains and isolates. A derived consensus sequence is shown above the alignment. Nucleotides identical to the consensus are indicated by dashes (-), nucleotides differing from the consensus are shown in small lettering. The CMV-Wem sequence is shown in full with the nucleotides differing from the consensus printed in bold.

Table 2.1 Percentage sequence differences between the coat protein nucleotide sequences of 15 cucumber mosaic virus strains and isolates. Sources of sequence data: see Materials and Methods.

	Virus strains and isolates														
	C	Chi	Wem	Fc	Fny	I17	Kin	O	P6	PR1	PR36	PR50	Q	WL	Y
CMV-C	-														
CMV-Chi	7.6	-													
CMV-Wem	**23.7**	**23.8**	-												
CMV-Fc	1.3	7.1	**23.2**	-											
CMV-Fny	0.4	7.1	**23.4**	0.9	-										
CMV-I17F	0.7	7.5	**23.0**	1.2	0.3	-									
CMV-Kin	23.7	23.6	**1.1**	23.2	23.4	23.0	-								
CMV-O	2.3	7.5	**23.7**	2.0	1.8	2.1	23.7	-							
CMV-P6	1.2	7.0	**23.7**	1.7	0.7	1.1	23.7	2.6	-						
CMV-Pr1	3.4	7.6	**22.9**	3.0	2.9	2.9	22.9	3.2	3.0	-					
CMV-PR36	2.9	7.6	**23.2**	2.6	2.4	2.5	22.9	3.0	2.9	1.7	-				
CMV-PR50	2.6	7.5	**22.5**	2.3	2.1	2.1	22.5	2.7	2.3	1.4	1.5	-			
CMV-Q	23.1	22.9	**1.4**	22.6	22.8	22.4	0.9	23.1	23.1	22.3	22.3	21.9	-		
CMV-WL	23.6	23.4	**1.4**	23.1	23.2	22.9	0.9	23.6	23.6	22.8	22.8	22.3	0.9	-	
CMV-Y	2.7	7.3	**23.6**	2.1	2.3	2.6	23.2	2.9	2.7	2.7	2.3	2.0	22.6	23.1	-

These data allow the assignment of CMV-Wem to subgroup II. There is a proline at amino acid position 129 of this mosaic-inducing strain, which is consistent with the prediction made by Shintaku (1991). The high degree of conservation between viruses within a subgroup is surprising given the differences in biological and physical properties.

Despite the divergence in the CP sequences of the viruses between the two subgroups, sequence conservation is observed in some regions. The region of greatest conservation is from Leu 92 to Asp 193: because of its high conservation this region is likely to play an important role in the higher order structure of the CP, and in the building of the virion particle (Hayakawa *et al.*, 1988; Quemada *et al.*, 1989)

These data indicate that (i) strains of the same virus isolated from different geographical locations over an extended period of time show very little difference in sequence in spite of the degeneracy of the genetic code; (ii) CMV strains in the same subgroup show great nucleotide and amino acid conservation, suggesting other possible, as yet unidentified, roles for the RNA molecule; and (iii) large differences in the biological/physical properties can be brought about by one or a few nucleotide/amino acid changes (Owen *et al.*, 1990).

2.3.4.2. TNV-F5P cDNA sequence

Prior to the generation of exonuclease III/S1 deletion constructs for sequencing the pSTNB5 clone, a deletion was made of a non-essential region immediately 5' to the CP ORF as defined by preliminary DNA sequencing and the published data (Meulewaeter *et al*, 1990) (Fig. 2.8A). Two sets of overlapping deletion mutants of the pSTN4 clone were selected, each extending through the entire insert region (Fig. 2.8B). The sizes of the deletion increments were in the order of 200 to 250 bp. Five overlapping deletion mutants were selected for sequencing with the forward primer DNA strand and three with the reverse primer. The nucleotide sequence of the 842 bp TNV-F5P CP cDNA is shown in Fig. 2.9.

Sequence analysis confirmed the presence of a 831 bp ORF, starting with an ATG (nt 13-15) and terminating with a TAG codon. Using the PRETTY programme of the GCG package, comparative nucleotide sequence alignment was done with the sequences of the two other published TNV CPs (Fig. 2.10).

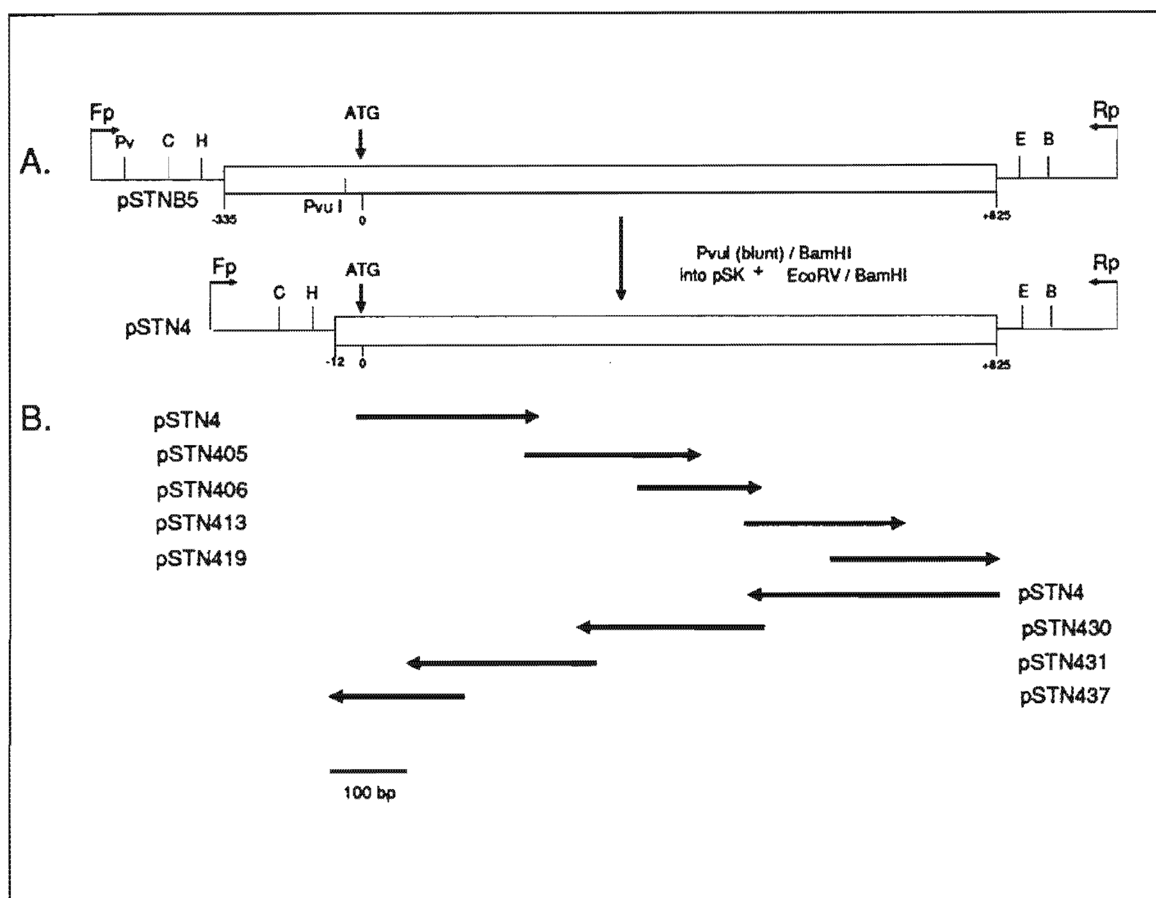

Fig. 2.8 Exonuclease III/S1 shortening of the pSTN4 clone to create two sets of overlapping deletion mutants **(B)** for nucleotide sequence analysis of the TNV-F5P CP ORF. Arrows represent the approximate number of base pairs read from each deletion mutant relative to the pSTN4 clone. **(A)** pSTNB5 was cut to completion with *Pvu*I, blunt-ended with T4 DNA polymerase and the largest 2.7 kb fragment gel purified with the Geneclean^R kit (BIO101, USA) and cut with *Bam*HI into two fragments. The smallest *Pvu*I(blunt)-*Bam*HI fragment was similarly gel purified and cloned into the *Eco*RV-*Bam*HI sites of Bluescript^R pSK$^+$ (Stratagene). Only selected restriction sites are indicated. B, *Bam*HI; C, *Cla*I; E, *Eco*RI, H, *Hin*dIII; Pv, *Pvu*I.

```
CGC AAA ATA GAC ATG GCA GGA AAG AAG AAC AAC AAC AAC GGT CAG   45
Arg Lys Ile Asp Met Ala Gly Lys Lys Asn Asn Asn Asn Gly Gln   11
        ⇐ P6] [CP ⇒
TAT ATA ATA CTG CGT ACA CCA GAG CAA CAG GTG GAG ATA GAC CAG   90
Tyr Ile Ile Leu Arg Thr Pro Glu Gln Gln Val Glu Ile Asp Gln   26

CGC AAC GCC CGT CGT GCT CAA ATG GGT CGC ATG AAA AAG GCT AGA  135
Arg Asn Ala Arg Arg Ala Gln Met Gly Arg Met Lys Lys Ala Arg   41

CAG CCC GTT CAG CGA TAC TTA CAA CAA CAC GGG TTG CAA AAC GGA  180
Gln Pro Val Gln Arg Tyr Leu Gln Gln His Gly Leu Gln Asn Gly   56

TTG TCC GGT AGA GGG GGC TAC ATA GTG GCT CCC ACT TCC GGG GGG  225
Leu Ser Gly Arg Gly Gly Tyr Ile Val Ala Pro Thr Ser Gly Gly   71

GTT GTC ACT CGA CCC ATA GTG CCG AAA TTC TCC AAC AGG GGA GAT  270
Val Val Thr Arg Pro Ile Val Pro Lys Phe Ser Asn Arg Gly Asp   86
```

```
TCC ACT ATA GTC CGT AAC ACT GAG ATC TTG AAC AAC CAG ATC TTA  315
Ser Thr Ile Val Arg Asn Thr Glu Ile Leu Asn Asn Gln Ile Leu  101

GCG GCA CTA GGC GCA TTC AAC ACA ACG AAC TTC GCA CTG ATT GCA  360
Ala Ala Leu Gly Ala Phe Asn Thr Thr Asn Phe Ala Leu Ile Ala  116

GCA GCA CCA TCG TGG CTG GCT GGC ATC GCT GAT CTT TAC AGC AAA  405
Ala Ala Pro Ser Trp Leu Ala Gly Ile Ala Asp Leu Tyr Ser Lys  131

TAT AGA TGG CTC TCA TGC GAG ATC ATC TAC ATA CCA AAA TGC CCC  450
Tyr Arg Trp Leu Ser Cys Glu Ile Ile Tyr Ile Pro Lys Cys Pro  146

ACC ACC ACC AGT GGA TCA ATT GCC ATG GCG TTC ACG TAC GAC AGA  495
Thr Thr Thr Ser Gly Ser Ile Ala Met Ala Phe Thr Tyr Asp Arg  161

AAC GAC GCT GCA CCC ACC ACA AGG GCT CAG TTG TCA CAA TCT TAT  540
Asn Asp Ala Ala Pro Thr Thr Arg Ala Gln Leu Ser Gln Ser Tyr  176

AAG GCC ATC AAT TTT CCA CCG TAT GCG GGA TAC GAC GGA GCA GCA  585
Lys Ala Ile Asn Phe Pro Pro Tyr Ala Gly Tyr Asp Gly Ala Ala  191

TAT TTG AAT TCG AAT CAG GGA GCT GGG TCT GCC ATT GCC GTT CAA  630
Tyr Leu Asn Ser Asn Gln Gly Ala Gly Ser Ala Ile Ala Val Gln  206

CTT GAC GTT ATC AAG TTG GAC AAG CCA TGG TAC CCC ACT ATC TCC  675
Leu Asp Val Ile Lys Leu Asp Lys Pro Trp Tyr Pro Thr Ile Ser  221

TCT GCC GGT TTC GGG GCG CTT GGC GTC CTC GAT CAG AAC CAA TTC  720
Ser Ala Gly Phe Gly Ala Leu Gly Val Leu Asp Gln Asn Gln Phe  236

TGC CCC GCG TCC CTC GTG GTC GCT AGC GAT GGG GGA CCC GCT ACT  765
Cys Pro Ala Ser Leu Val Val Ala Ser Asp Gly Gly Pro Ala Thr  251

GCT ACT CCA GCA GGG GAC CTT TTC ATC AAG TAC GTG ATT GAG TTC  810
Ala Thr Pro Ala Gly Asp Leu Phe Ile Lys Tyr Val Ile Glu Phe  266

ATT GAA CCA ATC GAC CCA ACA ATG AAC GTC TAG                  843
Ile Glu Pro Ile Asp Pro Thr Met Asn Val ***                  276
```

Fig. 2.9 Nucleotide sequence of TNV-F5P CP region. The predicted amino acid sequence is shown under the nucleotide sequence. Asterisks (***) indicates the stop codon. The primer binding sites for first-strand cDNA synthesis and PCR are underlined at the 3' and 5' ends of the sequence respectively.

The local South African isolate TNV-F5P is clearly similar to TNV-A (Meulewaeter *et al.*, 1990), with a 96% nucleotide sequence identity and 98% amino acid identity over the entire CP gene. The TNV-F5P CP amino acid sequence shows a six amino acid difference when compared with the TNV-A CP. Five out of the six are conservative substitutions of amino acids. Of the 31 nucleotide substitutions, 24 are in the third position of the codon resulting in silent mutations.

```
           1                                                 50                                                100
TNV-F5P    ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
TNV-A      ---------- ---------- ---------- ---------- ---------- t--------- ---------- ---------- ---------- ----------
TNV-D      ---c-taagc gagga-gagt tggattg-c- g-a-c-t-tc agtccaag-- -aaga----g a---a-a-tg a-t--a-cgc gtttcaga-a gagaaaatgg

Consensus  ATGGCAGGAA AGAAGAACAA CAACAACGGT CAGTATATAA TACTGCGTAC ACCAGAGCAA CAGGTGGAGA TAGACCAGCG CAACGCCCGT CGTGCTCAAA


         101                                                150                                                200
TNV-F5P    ---------- ---a------ ---------- ---------- ------a--- ---------- a--------- ---------- ---------- ----------
TNV-A      ---------- ---------- ---------- ---------- ---------- ---------- g--------- ---------- ---------- ----------
TNV-D      aac-agctc- -gc---taa- gcc-gcg-a- ca-caaagag t-ctgg-atg actttccgc- ccttaactg- tc-g-t-gct ---tctgtt- -ctatagcag

Consensus  TGGGTCGCAT GAAGAAGGCT AGACAGCCCG TTCAGCGATA CTTACAGCAA CACGGGTTGC -AAACGGATT GTCCGGTAGA GGGGGCTACA TAGTGGCTCC


         201                                                250                                                300
TNV-F5P    ---t------ ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----c----- ------g---
TNV-A      ---c------ ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ------a---
TNV-D      gc-acg--t- ccacag---c gcaccaa-ca ga--t-c-ct --tgtggt-- atact--g-t ggt-gctaat at--cattg- cagc-gc-gg agctttt-gt

Consensus  CAC-TCCGGG GGGGTTGTCA CTCGACCCAT AGTGCCGAAA TTCTCCAACA GGGGAGATTC CACTATAGTC CGTAACACTG AGATTTTGAA CAACCA-ATC


         301                                                350                                                400
TNV-F5P    ---------- ---------- ---c-----g ----t----- ---------- ---------g ---------g ---------- ---------- -----t----
TNV-A      --------g- ---------- ---------a ---------- ---------- ---------a ---------a ---------- ---------t -----c----
TNV-D      --ca-aa--- a-ccgttga- tcc--gtttt gga--ttggt -agcaaatat c--ggatctc -attctaaat -g-gatggat atca-gttc- gttgtgtata

Consensus  TTAGCGGCAC TAGGCGCATT CAATACAAC- AACTCCGCAC TGATTGCAGC AGCACCATC- TGGCTGGCT- GCATCGCTGA TCTTTACAGC AAATA-AGAT


         401                                                450                                                500
TNV-F5P    ---------- c--------- ---------- ---------- ---------- ---------- -------g-- ---g------ -----c---- ----------
TNV-A      ---------- ---------- -----t---- ---------- ---------- ---------- ---------- ---------- -----t---- ----------
TNV-D      ta-caaa--- -cct-cttct actca-gg-- gtgtggt--t gg-g-tagta tacgacgcac aagata--g- tc--actaca c-t-cac-a- t-t---aatg

Consensus  GGCTCTCATG TGAGATCATC TACATACCAA AATGCCCCAC CACCACCAGT GGATCAATTG CCATGGCTTT CACATACGAC AGAAA-GACG CTGCACCCAC


         501                                                550                                                600
TNV-F5P    -a-------- ---t------ ---------- ---------- ----------  ---------- ---------- ---------- ---------- ----------
TNV-A      -g-------- ---c------ -------c-- ---------- ----------  ---------- ---------- ---------- ---------- ----------
TNV-D      -tatcaat-c atcaca-tc- ctc-a---gc t-gata-gga ggag-ct-tg  cact-aacc- ta-g--tt-t -gtgg-gaat cg-t-gtgtc cacgttg-at

Consensus  C-CAAGGGCT CAG-TGTCAC AATCTTATAA GGCCATCAAT TTTCCACCGT  ATGCGGGATA CGACGGAGCA GCATATTTGA ATTCGAACCA GGGAGCTGGG


         601                                                650                                                700
TNV-F5P    --t-----t- ---------- ---c----t- ---------- ---------- ---------- ---------- -t-------- ---tg----- ----------
TNV-A      --a-----c- ---------- ---t----c- ---------- ---------- ---------- ---------- ---------- ----a----- ----------
TNV-D      a-caat-ga- tg-a-a---g atggtac-g- -cca-c-gt- -cg-tgct-t ta-tg--c-- a-a--aatt- a-aagaatca -t--t-tcca gc-acagct-

Consensus  TC-GCCAT-G CCGTTCAACT TGA-GTTA-C AAGTTGGACA AGCCATGGTA CCCCACTATC TCCTCTGCCG GCTTCGGGGC GCTC-GCGTC CTCGATCAGA
```

```
          701                                                 750                                                800
TNV-F5P   ---------- ---------- --c------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
TNV-A     ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
TNV-D     ttattgctg- ggat-gagga -c--ct-ct- -c-ct-c--t c---gatat- ttcatgcgat ac-acatc-- gt-ca-tgaa cc-gt-aatc cctcca---a

Consensus ACCAATTCTG CCCCGCGTCC CTTGTGGTCG CTAGCGATGG GGGACCCGCT ACTGCTACTC CAGCAGGGGA CCTTTTCATC AAGTACGTGA TTGAGTTCAT

          801                        831
TNV-F5P   ---------- g--------- ---------- -
TNV-A     ---------- a--------- ---------- -
TNV-D     --ttta-... .......... .......... .

Consensus TGAACCAATC -ACCCAACAA TGAACGTCTA G
```

Fig. 2.10 Alignment of the TNV-F5P coat protein sequence with the two other sequenced TNV strains. A derived consensus sequence is shown below the alignment. Nucleotides identical to the consensus are indicated by dashes (-), nucleotides differing from the consensus are shown in small lettering.

Table 2.2 Percentage sequence differences between the coat protein nucleotide sequences of TNV-A, TNV-D and TNV-F5P. Sources of sequence data are: TNV-A; Meulewaeter *et at.*, 1990, TNV-D; Coutts *et al.*, 1991, TNV-F5P this chapter.

Virus strains and isolate			
	TNV-F5P	TNV-A	TNV-D
TNV-F5P	-	3.9	47.5
TNV-A		-	46.5
TNV-D			-

The sequence of the 29 kDa TNV-D CP (Coutts *et al.*, 1991) does not show the same striking similarity with either the 30 kDa TNV-F5P and TNV-A CPs. A comparison of the nucleotide and amino acid sequence of TNV-F5P and TNV-D shows an overall 49% identity and very limited identity in the a and R domains. However there is a 59% nucleotide identity within the S domain. This is consistent with the literature, and although this similarity is not as great as that of TNV-F5P and TNV-A, it is interesting to note that TNV-A and TNV-D are serologically related (Coutts *et al.*, 1991).

CHAPTER 3

Subcloning the CMV and TNV CP genes into plant expression vectors

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CHAPTER 3

Subcloning the CMV and TNV CP genes into plant expression vectors

SUMMARY

To study resistance against local South African isolates of CMV and TNV in transgenic tobacco plants, the CP cistrons of isolates of both viruses were individually cloned in both orientations (sense and antisense) in *Agrobacterium tumefaciens* Ti-plasmid-based binary or cointegrate vectors. The study was then extended to include engineering doubly transgenic plants. For this purpose the hygromycin resistance marker was cloned into a plasmid harbouring the TNV CP gene disrupting the kanamycin resistance marker, thus enabling secondary transformation of a CMV CP+ transformant, by selection on kanamycin and hygromycin. A truncated form of the TNV CP was generated by deleting 83 amino acids from the C-terminal region (S-domain): this was done to determine whether in this case the full-length CP is required to mediate virus resistance.

3.1 INTRODUCTION

3.1.1 Binary and cointegrate vector systems

The biology of *A. tumefaciens* is described in Chapter 5.

The transformation vectors used with *A. tumefaciens* are divided into two types, cointegrate or binary vectors. Both are based on the fact that during T-strand transfer the *vir* region acts in *trans*, and DNA that is located between the 25 bp border repeats is transferred from the Ti plasmid to the plant genome (for a review see Ream, 1989).

Cointegrate vectors are derivatives of a wild-type Ti plasmid from which T-DNA-encoded tumour genes have been replaced by pBR322-related cloning vector sequences. Foreign genes cloned into pBR322-derived intermediate vectors are mobilized from *E. coli* into *A. tumefaciens*, and integrate into the acceptor Ti plasmid by a single cross-over between homologous pBR322 sequences of the Ti plasmid and the intermediate vector.

Binary vectors contain origins of replication and selectable markers functional in *E. coli* and *A. tumefaciens* and hence can be maintained in both types of bacteria. These vectors contain the 25 bp border repeats between which are located appropriate cloning sites for the insertion of foreign DNA and selectable markers functional in plant cells. The *A. tumefaciens* host for plant transformation with a binary vector harbours a Ti plasmid from which the T-DNA has been deleted.

Cointegrate vectors have the advantage that they are generally stable in *A. tumefaciens* (Walden and Schell, 1990). Binary vectors tend to be unstable in *A. tumefaciens* and require selection to confirm their maintenance. By far the most important feature of binary vector systems is their host range. It appears that by selecting an appropriate *A. tumefaciens* chromosomal background and helper Ti plasmid, the optimal culture conditions, and appropriate plant tissues, most higher dicotyledons will be amenable to efficient, binary vector-mediated transformation (An *et al*, 1988).

3.1.2 Description of the plant expression vectors used

To enable CMV and TNV CP expression in transgenic plants the CP cistrons were cloned into *A. tumefaciens* Ti-plasmid based plant expression vectors. Three vectors were used in this study: a cointegrate vector, pGSJ280 (gift from Plant Genetic Systems, Gent; Deblaere *et al.*, 1987), and two binary vectors, the β-glucuronidase reporter plasmid, pBI121 (CLONTECH Laboratories, Inc; Jefferson *et al.*, 1987), and an expression vector for the efficient transformation of cucurbits, pGA643 (An *et al.*, 1988).

pGSJ280 (Fig. 3.1) is based on the common *E. coli* cloning vector, pBR322, and can be directly mobilized into *A. tumefaciens* strain C58C1Rif^R (pGV2260) (Deblaere *et al.*, 1985). pGV2260 is a derivative of the octopine Ti plasmid, pTiB6S3, in which the entire T-region including the 25 bp (both T_L and T_R) border sequences have been deleted and replaced by pBR322 sequences, including the β-lactamase gene (Cb^R). The pBR322 sequence in pGSJ280 contains a *bom* site, and mobilization as well as transfer functions which can be complemented *in trans* (Deblaere *et al.*, 1987). Since pGSJ280 cannot replicate in *A. tumefaciens* it will be maintained only if recombination occurs between the homologous pBR322 sequences on it and pGV2260. This results in the formation of a cointegrate in which the T-DNA is flanked by directly repeated pBR322 sequences.

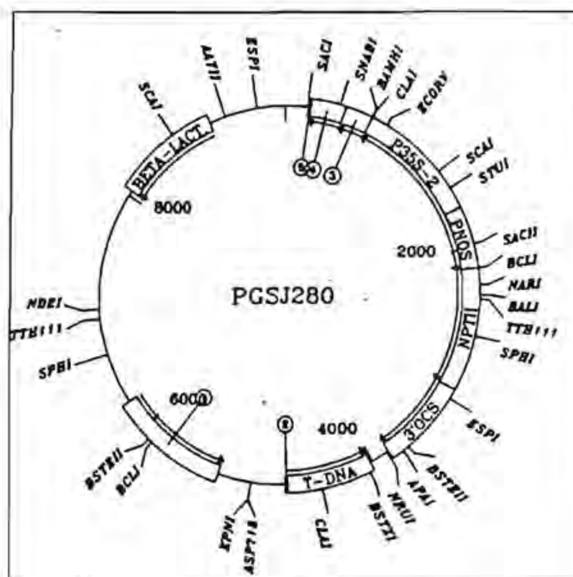

Fig. 3.1 Schematic representation of the pGSJ280 co-integration vector, showing the two unique restriction sites, *Bam*HI and *Cla*I between the CaMV 35S promoter and the polyadenylation fragment from T_L-DNA gene 7 (T7). Labels 1, Streptomycin-Spectinomycin resistance marker. 2, Left border sequence. 3, 3'-end T7. 4, T-DNA. 5, Right border sequence.

The general purpose binary plant expression vector pBI121 (Fig. 3.2) was made by transferring the coding region of GUS (β-glucuronidase) and the cauliflower mosaic virus (CaMV) 35S promoter into the polylinker site of pBIN19 (Jefferson *et al*., 1987). The broad host range origin of replication allows for replication in both *E. coli* and *A. tumefaciens*. The pBIN19 binary system (Bevan, 1984) utilises the *trans*-acting functions of the *vir* region on a resident disarmed Ti plasmid to transfer the modified T-DNA region from the small autonomous replicon in *A. tumefaciens* to plant cells. The modified T-DNA contains only border repeats, a selectable marker, in this case the neomycin phosphotransferase gene (*npt*II) and a multiple cloning site for the insertion of sequences for transfer. Thus, unlike the cointegrate vector system, binary vectors require no recombinational steps for integration into the Ti plasmid. This feature becomes advantageous when intrinsically unstable sequences are to be transformed.

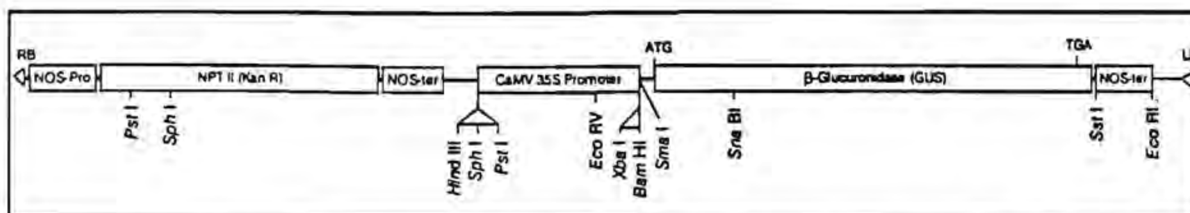

Fig. 3.2 Schematic representation of the T-DNA region of the pBI121 binary vector, showing the unique polylinker cloning sites *Xba*I, *Bam*HI and *Sma*I, between the CaMV 35S promoter and the GUS gene. The GUS gene is followed by the nopaline synthase polyadenylation site (NOS-ter). Figure taken from CLONETECH manual.

pGA643 (Fig. 3.3) is an expression vector that contains, in addition to the general elements of the binary vectors, a DNA fragment carrying the transcript 7 and 5 terminators of the octopine-type Ti plasmid, pTiA6, and a 419 bp DNA fragment carrying the 35S promoter of CaMV (An *et al.*, 1988).

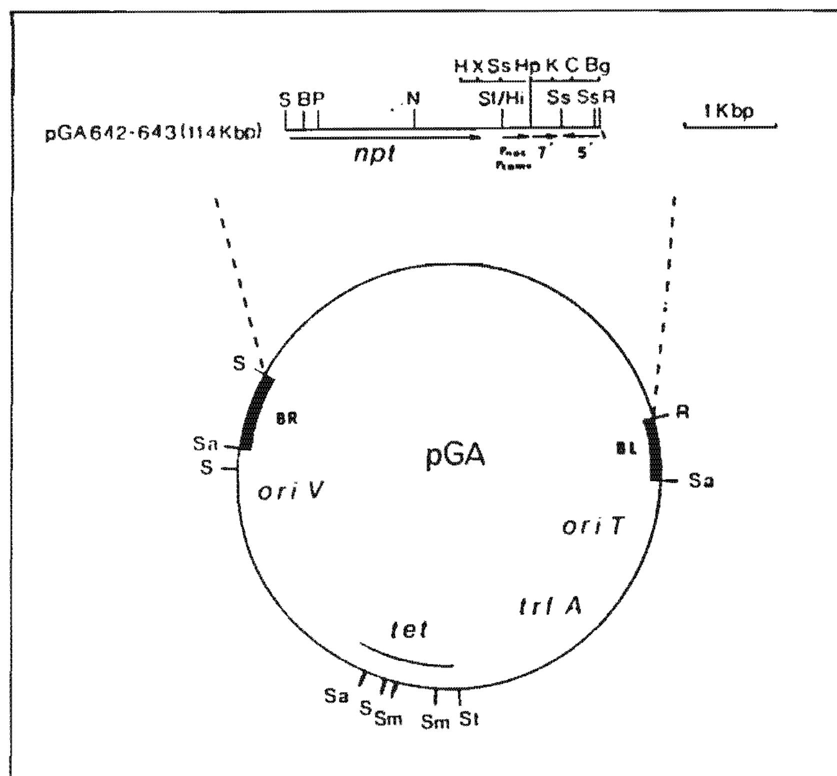

Fig. 3.3	Schematic representation of the T-DNA transfer region of the pGA643 binary expression vector. This expression vector has six polylinker cloning sites between the CaMV 35S promoter and the transcription termination (polyadenylation) region of gene 7 of pTiA6. The *SstI* site in the polylinker is not unique. The wide host range replicon required for maintenance of the vectors in *E. coli* and *Agrobacterium*, as well as the fragments containing the right and left border sequences, are shown in the circular map. Restriction sites: Bg, *BglII*; C, *ClaI*; H, *HindIII*; Hp, *HpaI*; K, *KpnI*; Ss, *SstI*; X, *XbaI*. Figure adapted from An *et al.*, 1988.

3.1.3 Expression of multiple traits in transgenic plants

It has been demonstrated that it is possible to introduce multiple traits, such as resistance to two different viruses, into plants. The first example of dual virus resistance was the expression of potato virus X (PVX) and potato virus Y (PVY)

CP genes in a commercial cultivar of potato (Lawson *et al.*, 1990). In this approach the two genes were cloned together into one plant expression vector and transformed into a plant simultaneously. Thus the multiple genes are linked together and inserted into the chromosome at the same locus. Yie *et al.* (1992) employed the same approach in expressing CMV Sat-RNA and the CMV CP gene in tandem. A second approach is to clone each gene into a plant expression vector and transform plants separately: transgenic plants stably inheriting and expressing multiple genes are then obtained by crossing. We have employed a third approach by which each gene is cloned into a separate expression vector carrying a different selectable marker. Secondary transformation can be carried out using the primary transgenic plants which have stably inherited the first gene, selecting for the dual antibiotic resistance phenotype.

3.1.4 "Pathogen-derived resistance" to virus infection

The theory of pathogen-derived resistance (Sanford and Johnson, 1985) predicts that a "normal" host-pathogen relationship can be disrupted if the host organism expresses essential pathogen-derived genes. It has been proposed that host organisms expressing pathogen gene products in excess amounts, at the inappropriate developmental stage or in a dysfunctional form, may disrupt the normal replicative cycle of the pathogen and result in an attenuation or aborted infection of the host. It has been demonstrated that transgenic plants expressing a plant virus CP can be resistant to infection by the homologous virus or a related strain. This type of pathogen-derived resistance is known as "CP-mediated resistance" and has been demonstrated for a growing number of economically important plant viruses (for a review see Chapter 1).

It has previously been shown that "dysfunctional" truncated forms of the tobacco etch virus (TEV) CP tended to confer greater protection than the full-length CP (Lindbo and Dougherty, 1992). A highly basic amino-terminal (N-terminal) arm is a common feature for CPs of several RNA-containing spherical and bacilli form plant viruses. This seems to be the end of the CP molecule which interacts with the viral RNA in the virus interior (Erickson and Rossman, 1982). This proposal was verified in a study by Zuidema *et al.* (1984), in which the first 25 amino acid residues were removed from the N-terminus of the alfalfa mosaic virus CP. This resulted in loss both of its ability to activate the genome and to bind at specific sites on the RNA molecule.

The most plausible hypothesis proposed to explain the basis of CP-mediated resistance entails the encapsidation, or blockage of uncoating of the challenging virus genome, by free CP expressed in transgenic plants (reviewed in Chapter 1). Taking this into account, and the fact that TNV CP has a basic N-terminal arm, it was decided to remove a portion of the C-terminal end of the CP. This construct could then be used to determine whether or not this truncated form of the TNV CP is sufficient to mediate virus resistance.

This chapter reports the manipulation and subcloning of the CMV and TNV CP genes in both orientations in plant expression vectors. In the case of TNV a truncated form of the CP was similarly cloned. The resulting constructs were subsequently used for tobacco leaf disc transformations (described in Chapter 5).

3.2 MATERIALS AND METHODS

Methods and techniques not described or referenced here were performed as described by Sambrook *et al.*, 1989.

3.2.1 Isolation of DNA fragments from agarose gels

Restriction enzyme-generated DNA fragments were separated from plasmid vector by agarose gel electrophoresis, and gel purified using the Geneclean^R (Bio101, U.S.A.) method (as described by the distributors). Small DNA fragments, up to 350 bp, were isolated from agarose by the "freeze-squeeze" method (Tautz and Renz, 1983).

3.2.2 PCR amplification of the TNV CP gene

PCR amplification reactions contained 25 pmol (100 ng) of each primer and 15 ng of *Cla*I linearised template DNA (pSTN4 carrying the TNV CP gene) in a total reaction volume of 50 µl. DMSO was added to a final concentration of 10% and 200 µM of each nucleotide used. Promega buffer and enzyme was used. Reaction mixtures were subject to 30 cycles of amplification using a denaturation step of 93°C (30 sec), annealing step of 42°C (40 sec), and an extension step of 72°C (1 min). DNA amplification cycles were controlled by a custom-made programmable thermocycler (JDI Model 8012). After amplification, samples were electrophoresed through a 0.8% agarose gel and the correct size band eluted using the Geneclean^R procedure.

3.2.3 Analysis of recombinant clones by hybridization

Recombinant clones were screened for the presence of the insert fragment using the colony hybridization method of Buluwela *et al*, 1989. DNA for probing was radiolabelled with [α-^{32}P]dCTP by nick translation, using an Amersham kit (N.5000, Amersham, UK), as described by the distributors. Nonradioactive labelling and hybridization procedures using digoxigenin-11-dUTP (DIG)-DNA probes was performed as described by the distributor (Boehringer Mannheim).

3.3 RESULTS AND DISCUSSION

3.3.1 Subcloning the sense and antisense CMV CP gene constructs into two plant expression vectors

Prior to subcloning the CMV CP gene into the plant expression vectors pGSJ280 and pGA643, a 912 bp fragment from pCDCR379 (see Chapter 2) was subcloned into the blunt *EcoR*V site of pBluescript^R pSK$^+$ and pKS$^+$ (Stratagene, La Jolla, CA). The CMV CP coding insert, including the 143 bp portion of the 5' non-coding region and a 97 bp portion of the 3' non-coding region, was excised from pCDCR379 using the restriction enzymes *BamHI* and *Sac*I. The resulting 5' and 3' protruding ends were filled in using T4 DNA polymerase and the fragment ligated into the pBluescript^R SK$^+$ and KS$^+$. Recombinant plasmids were transformed into *E. coli* LK111 competent cells. The presence of the subcloned fragment was verified by restriction enzyme digestion and agarose gel electrophoresis, resulting in plasmids pSK1 and pKS18 (Fig. 3.4A). A construct carrying all three start codons (Fig 3.4A.1) was created by subcloning the 912 bp *Bam*HI-*Cla*I CMV CP coding fragment from pKS18 (Fig. 3.4A.2) into the same sites of the cointegrate vector pGSJ280 (Deblaere *et al.*, 1987). This resulted in plasmid pGSC315 (Fig. 3.4B).

Two antisense CMV CP constructs were made. The first by subcloning the 912 bp *Bam*HI-*Cla*I fragment from pSK1 into the *Bgl*II and *Cla*I sites of the binary vector pGA643, and the second by subcloning the same fragment into the *Bam*HI-*Cla*I sites of pGSJ280. This resulted in plasmids pGCMA (not shown) and pGAC340 (Fig. 3.4B) respectively.

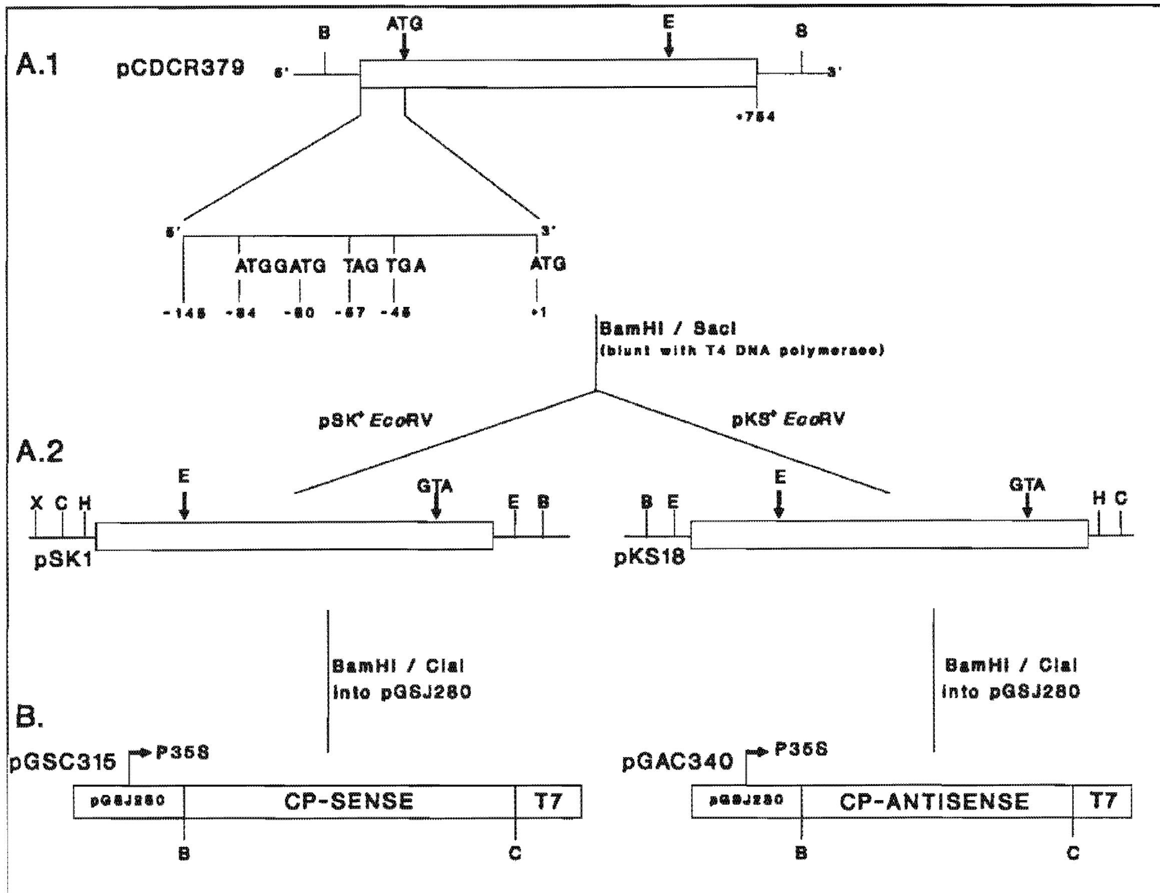

Fig. 3.4 Construction of the *Agrobacterium* cointegration vectors pGSC315 (CP-sense) and pGAC340 (CP-antisense) carrying the CMV-Wem CP gene inserted in both orientations with respect to the CaMV 35S promoter [B]. [A.1] Schematic representation of the insert in pCDCR379 showing the two ATG start codons at positions -84 and -80 and the two stop codons at positions -57 and -45 in the 5' untranslated region. [A.2] The intermediate vectors pSK1 and pKS18 were constructed as described in the text. Only selected restriction sites are indicated and the plasmids are not drawn to scale. Restriction sites: B, *Bam*HI; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; S, *Sac*I; X, *Xba*I.

Gene expression is expected to be influenced by the efficiency of ribosomal binding. The absence of a consensus ribosome binding site in eukaryotic viral and cellular mRNAs led Kozak to propose the ribosome scanning model for translational initiation (Rogers *et al.*, 1985; Kozak, 1986). This model proposes that the eukaryotic 40S ribosome subunit binds at the cap site of the messenger RNA and scans along it until it reaches an ATG start codon whereupon the 60S subunit binds and translation proceeds. One prediction of this model is that ATG signals in the mRNA preceding the ATG initiator of the protein of interest will impair the expression of that protein by causing translation of a nonsense protein and decreasing the frequency of correct starts.

Evidence from the sequence data obtained earlier (see Chapter 2, section 2.3.4.1) showed the presence of two ATG start codons and two stop codons in the sequence 5' of the start of the CP gene (Fig. 3.4A.1). These had to be removed in order to express the CMV CP gene in plants and in *E. coli*. Due to the lack of unique restriction sites in the sequence the insert was subjected to unidirectional shortening using exonuclease III and S1 nuclease digestion (Henikoff, 1984). pSK1 was first cut with *BstX*1 leaving a 3' overhanging exonuclease-protected end. Exonuclease digestion progressed from the 5' overhang created by a *BamH*1 restriction digestion. Deletion mutants were initially selected on the basis of size by *Pvu*II digestion and agarose gel electrophoresis. A few select deletion mutants which appeared to have had the required number of nucleotides removed were then sequenced. Sequence analysis of a number of putative clones shortened to the correct length finally yielded plasmid pSCM9 (Fig. 3.5A.1), harbouring a 800 bp insert fragment with a 47 bp 5' non-coding region.

The insert in pSCM9, which had both start codons but only one stop codon removed, was selected for subcloning into the plant expression vectors. In order to make use of extra polylinker sites which were lost in the shortening, the blunt-ended *Sac*I-*Cla*I CMV-CP insert from pSCM9 was first cloned in the correct orientation into the dephosphorylated *Eco*RV site of pBluescript^R SK$^+$. This resulted in plasmid pS15 (Fig 3.5A.2).

The sense CMV CP construct in the binary vector pGA643 (An *et al.*, 1988) was created by subcloning the 866 bp CP-encoding fragment from pS15. The fragment was excised using *Cla*I and *Xba*I, and ligated into the same sites in pGA643 between the CaMV 35S promoter and the transcription terminator of the octopine-type Ti plasmid pTiA6 to generate pGCMS (Fig. 3.5B).

The presence and orientation of the inserts in the recombinant plasmids was confirmed by colony or dot-blot analysis and by restriction enzyme mapping (results not shown).

3.3.2 Subcloning the sense and antisense TNV CP gene constructs into two plant expression vectors

Sequence analysis of the TNV CP gene in plasmid pSTN4 (see section 2.3.4.2) indicated that the penultimate codon and the stop codon were not present. This problem could not be solved by manipulation of restriction sites in order to

create a stop codon required for plant or *E. coli* expression. PCR-mediated oligonucleotide mutagenesis was therefore carried out using the primer 5'-CCGGATCCCTAGACGTTCATTGTTGGGTTG-3', complementary to sequence 822 to 843 of TNV-F5P at the 3' end (Fig. 2.9). This primer includes a *Bam*HI site plus two extra nucleotides on the 3' end. The primers complementary to the 5' end, were either the vector-specific KS primer, or the TNV genome-specific primer, 5'-GACATGGCAGGAAAGAAG-3', complementary to sequence 10 to 27 of the TNV-F5P.

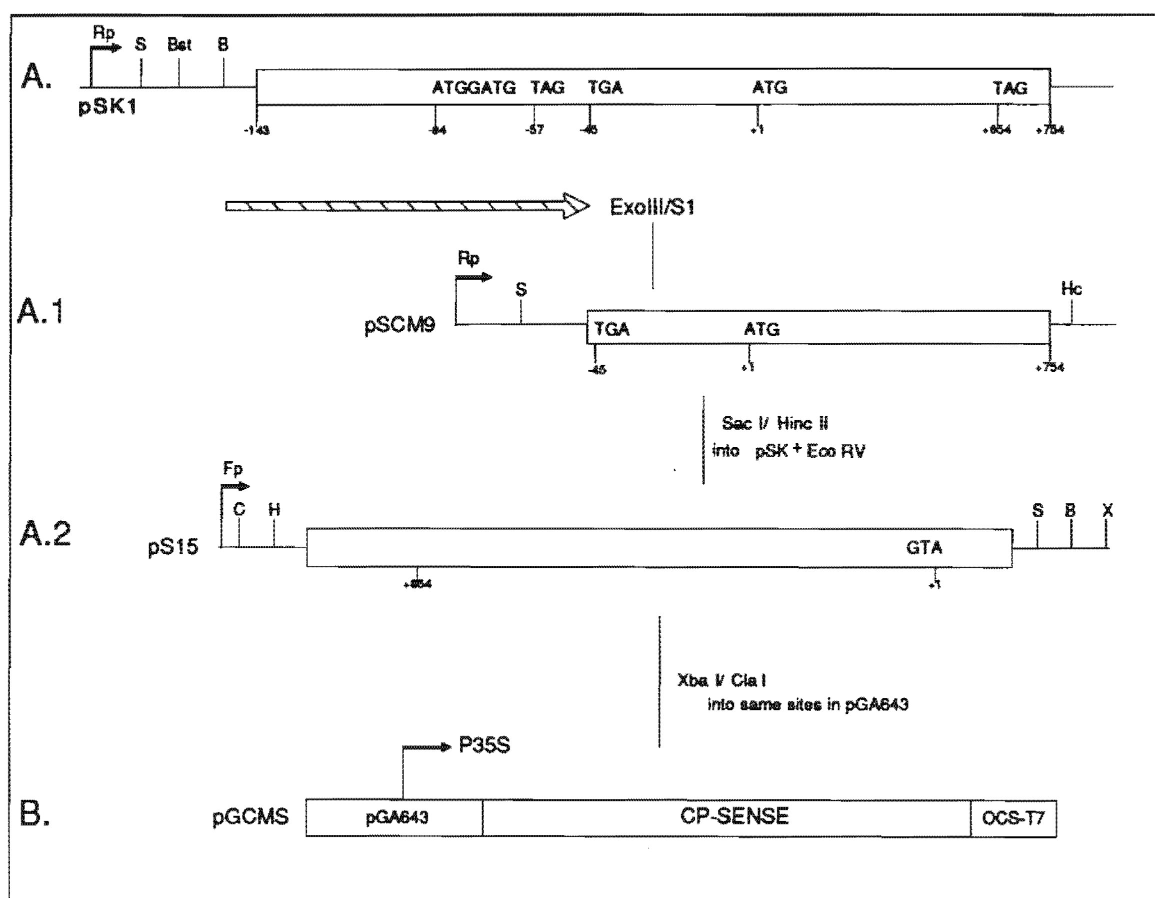

Fig. 3.5 Construction of *Agrobacterium* binary vector pGCMS (CMV CP-sense) [B] carrying the exonucleaseIII/S1 treated CP gene lacking the two ATG start codons and one stop codon, cloned between the CaMV 35S promoter (P35S) and the T7 transcription terminator (OCS-T7) of pGA643 as described in the text (section 3.3.1). [A.1] The direction of the large open arrow shows the direction of exonuclease digestion generating the deletion mutant pSCM9. [A.2] The intermediate vector pS15. Only selected restriction sites are indicated and the plasmids are not drawn to scale. Restriction sites: B, *Bam*HI; Bst, *Bst*XI; C, *Cla*I; Hc, *Hinc*II; H, *Hind*III; S, *Sac*I; X, *Xba*I.
R_p: Reverse sequencing primer with the direction of sequencing indicated by a closed arrow.
F_p: Forward sequencing primer.

The PCR-generated fragment was then cut with *Bam*HI and ligated into pBluescript SK+ which had been cut with *Eco*RV, dephosphorylated, and cut with *Bam*HI. Putative positive recombinants were selected on the basis either of restriction enzyme mapping or colony hybridization, followed by DNA sequence analysis.

Unfortunately, PCR oligonucleotide mutagenesis did not yield a recombinant clone harbouring the TNV CP coding region with a stop (TAG) codon. The PCR reaction reliably gave a band of 835 bp in length (Fig. 3.6). A number of alternative cloning methods, (Lorens, 1991; Marchuk *et al.*, 1991; Liu and Schwartz, 1992) however, failed to give the correct size insert. This could be due to random, non-specific priming during the PCR reaction, or nicking of the resultant PCR DNA during gel purification.

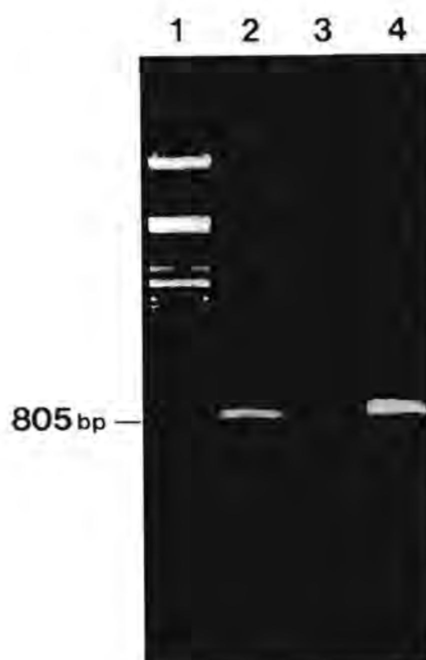

Fig. 3.6 Agarose gel electrophoresis of TNV CP and CMV CP PCR products. Lane 1, λ (*Pst*I). Lane 2, CMV CP PCR product amplified from pSCM9. Lane 3, pSK+ PCR control. Lane 4, 835 bp TNV CP PCR product amplified from pSTN4.

A stop codon was successfully introduced by cDNA cloning using the same 3' synthetic oligonucleotide primer described above. The resulting cDNA fragments were cloned into pBluescript SK+ essentially by the same method as described in section 2.2.3.

Insertion of the blunt-ended cDNA fragments into the dephosphorylated *Eco*RV site of pBluescript^R SK$^+$ resulted in seven positive recombinants based on colony hybridization. Restriction analysis confirmed the presence of the inserts and orientation with respect to the *Eco*RI site at the 3' end of the TNV CP gene. A single recombinant, pTNV-cDNA7, harboured a TNV cDNA clone in the correct orientation (Fig. 3.7A.1). Sequencing more than 250 bp from the 3' end of this plasmid confirmed a 100% sequence similarity with the original TNV-F5P cDNA clone in pSTN4. As reverse transcriptase has no editing function, it cannot be assumed that any two cDNA clones are the same. Therefore in order not to have to sequence an entire new clone, the *Eco*RI fragment from pTNV-cDNA7 (which by sequence analysis was identical to the same fragment of the original pSTN4 clone) was subcloned into the *Eco*RI-digested pSTN4 plasmid. The 269 bp *Eco*RI fragment was isolated from a 1% agarose gel by the "freeze-squeeze" procedure. The fragment lacking the stop codon was deleted from pSTN4 by electrophoresis of the *Eco*RI-digested plasmid DNA and isolation of the large vector-insert band using the Geneclean^R method (BIO 101, USA). This was dephosphorylated to reduce recirclarization. The ligation reaction was transformed into *E. coli* JM105 and recombinant clones selected by *Eco*RI restriction analysis. Orientation of the cloned fragment was confirmed by PCR, and the presence of the TAG stop codon once again verified by sequence analysis. This subcloning produced the vector pTAG1 (Fig. 3.7A.2).

To construct TNV CP plant expression vectors the 898 bp CP-coding *Hinc*II-*Bam*HI and *Cla*I-*Bam*HI fragments from pTAG1 were subcloned into the appropriately restricted pBI121 and pGA643 vectors to generate pBITS1 and pGAT12 respectively (Fig. 3.7B). pBI121 was prepared by cutting with *Xba*I, filling-in with T4 DNA polymerase, and then cutting with *Bam*HI. The *Bam*HI-*Hinc*II TNV CP coding fragment was cut with *Cla*I to generate a *Bam*HI-*Cla*I fragment and cloned into the *Bgl*II-*Cla*I sites of plasmid pGA643, generating pGAT12. The presence and orientation of the TNV CP sequence in the correct (sense) orientation with respect to the CaMV 35S promoter, was determined by colony hybridization and restriction mapping (results not shown).

A third TNV CP construct was made by inserting the same *Hinc*II-*Bam*HI fragment, as described above, into the *Bam*HI-*Sma*I sites of pBI121 generating pBITA45 (Fig. 3.7B). This plasmid has the TNV CP sequence in the reverse (antisense) orientation with respect to the CaMV 35S promoter.

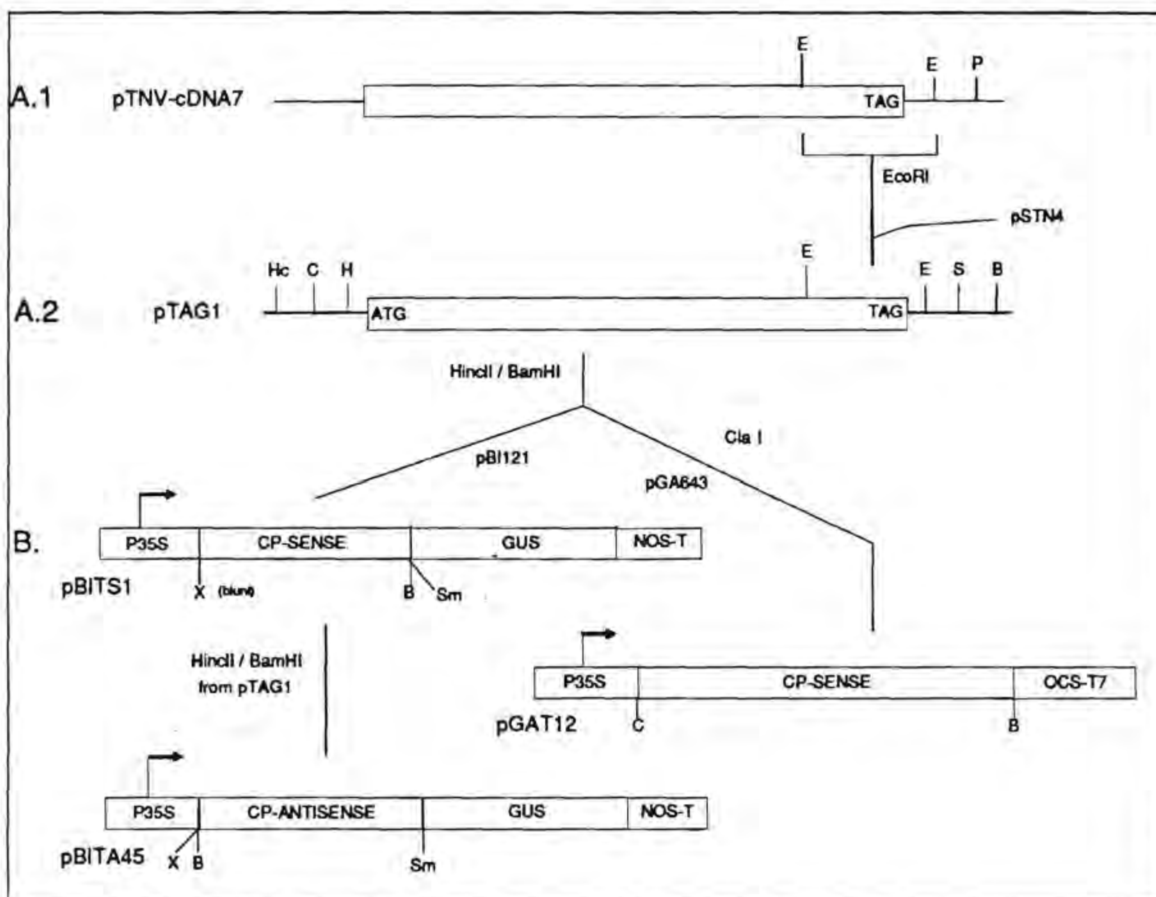

Fig. 3.7 Construction and schematic representation of *Agrobacterium* binary vectors pBITS1 (TNV CP-sense), pBITA45 (TNV CP-antisense), pGAT12 (TNV CP-sense) with the TNV-F5P CP gene inserted in both orientations between the CaMV 35S promoter and a transcription terminator region **[B]**. **[A.1]** This shows a map of pTNV-cDNA1 with the correct 3' sequence, including a translational stop codon, and the position of the *Eco*RI sites used for subcloning the *Eco*RI fragment into the same sites in pSTN4. This subcloning, in the correct orientation, placed the fragment in the same translation frame and resulted in plasmid pTAG1 **[A.2]**. The cloning strategy is described in detail in the text (Section 3.3.2). Only selected restriction sites are indicated and the plasmids are not drawn to scale. Restriction sites: B, *Bam*HI; C, *Cla*I; E, *Eco*RI; H, *Hinc*II; H, *Hind*III; P, *Pst*I; S, *Sac*I; Sm, *Sma*I; X, *Xba*I.

3.3.3 Subcloning the hygromycin resistance gene into pGAT12

The hygromycin resistance (Hyg^R) selectable marker was introduced into pGAT12, harbouring the TNV CP coding region, allowing the use of this vector to transform leaf discs from transgenic Km^R plants in order to generate secondary transformants.

The hygromycin gene was excised from the pAK1 vector (kindly donated by C. Lichtenstein) as a *Kpn*I fragment (Fig. 3.8A) and made blunt with T4 DNA polymerase. The introduction of this 2.5 kb fragment into the unique *Bam*HI site

(also made blunt), positioned at the 5' end of the *npt*II gene of pGAT12, disrupted the Km^R marker. Medium containing 20 µg/ml Km did not support LK112 (pGAT12-H4) growth, confirming the disruption of the gene. The insertion of the Hyg^R gene was not orientation dependent as this gene is bordered by an upstream CaMV 35S promoter and a downstream octopine synthase termination signal (Fig. 3.8B). The presence of the insert was confirmed by restriction mapping (results not shown).

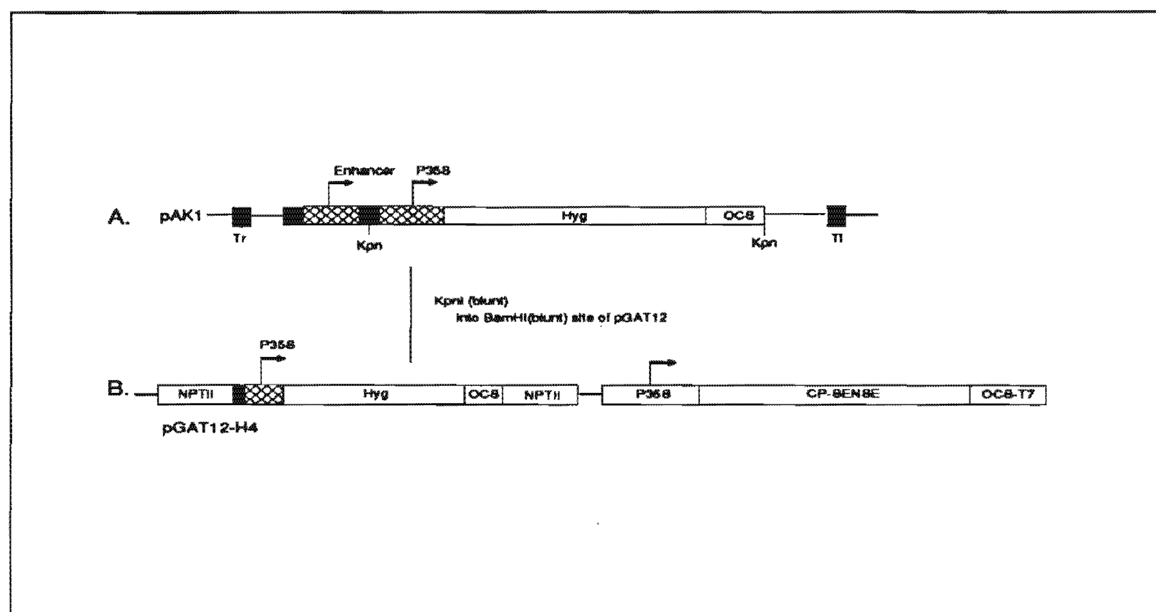

Fig. 3.8 Construction of the hygromycin resistance encoding plasmid pGAT12-H4. [A] Schematic representation of the T-DNA transfer region of the binary vector pAK1, containing the hygromycin resistance gene (Hyg) situated between a CaMV 35S promoter-enhancer region and a octopine synthase terminator region (OCS). [B] The construction of the *Agrobacterium* binary vector pGAT12-H4 containing a dysfunctional neomycin phosphotransferase II gene (NPTII) due to insertion of the Hyg^R gene into the *Bam*HI site of pGAT12 as described in the text. Tl/Tr: T-DNA left and right border integration sequences. Restriction site: Kpn, *Kpn*I.

3.3.4 Construction of a truncated TNV CP gene

The objective of this construction was to truncate the TNV CP gene in order to express in plants only what is probably the equivalent, in the TNV CP, of the N-terminal (or R domain), the (a) domain and N-terminal half of the S domain of the CP (see section 2.1.2). By challenging the TNV CP-expressing transgenic plants and the truncated TNV CP expressing transgenic plants with TNV, a conclusion could be drawn as to whether or not the entire CP is required to mediate virus resistance.

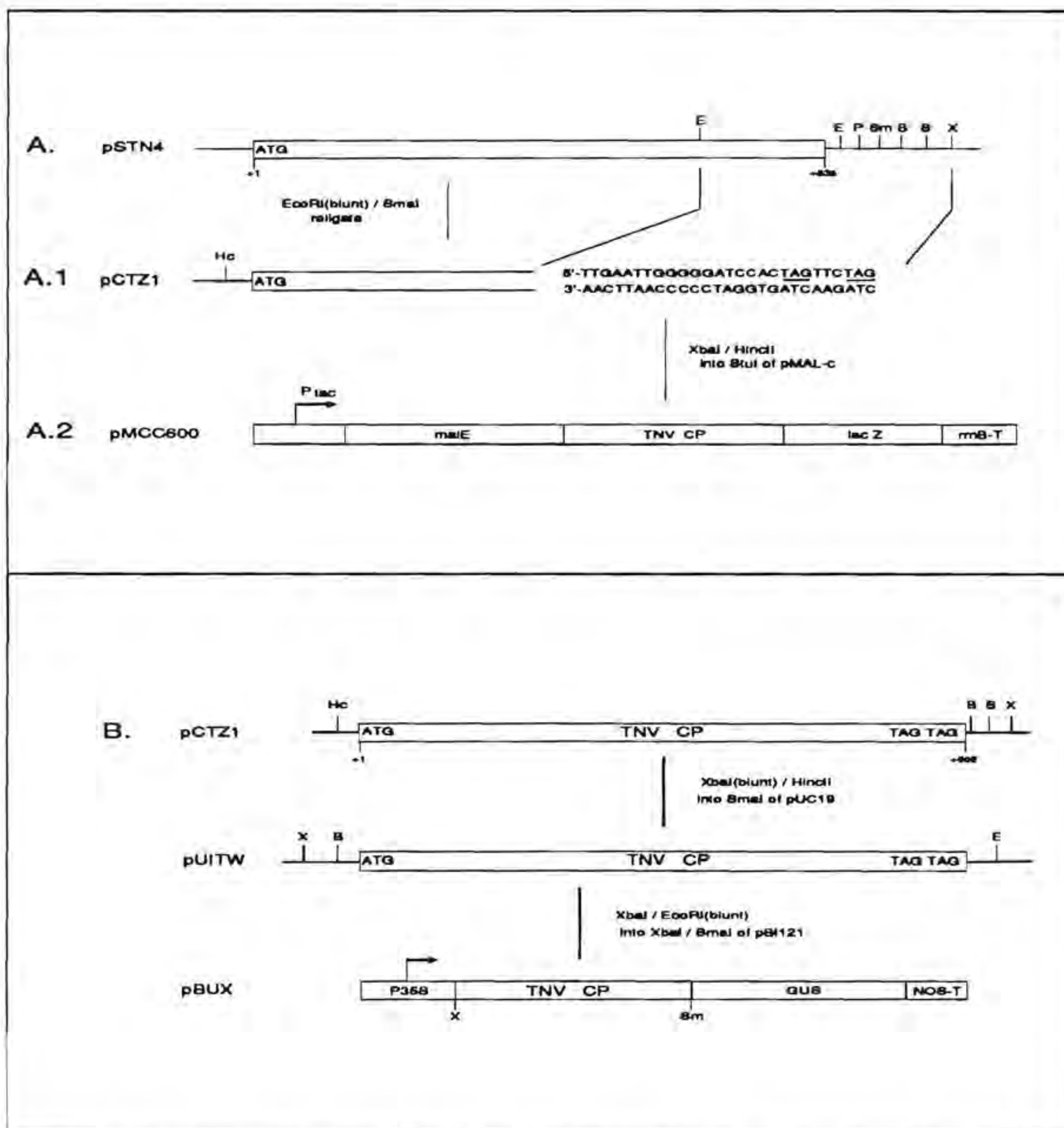

Fig. 3.9 Construction of a truncated TNV CP gene. **[A]** The deletion of a 294 bp *Eco*RI-*Sma*I fragment from pSTN4, resulting in pCTZ1 **[A.1]** with two in-frame TAG stop codons (underlined in the sequence shown). **[A.2]** The subcloning of the truncated TNV CP *Xba*I (blunt)-*Hinc*II fragment into the *Stu*I site of the *E. coli* expression vector pMAL^{TM}-c (New England BioLabs). This subcloning placed the fragment in the same translation frame as the maltose binding protein gene (*mal E*), resulting in a functional translation fusion. [B] Construction of the *Agrobacterium* binary vector pBUX with the truncated TNV CP gene cloned in the sense orientation, between the CaMV 35S promoter and nos transcriptional terminator region of pBI121 as described in the text. The truncated TNV CP gene fragment was first subcloned into the *Sma*I site of pUC19, resulting in pUITW, to facilitate directional cloning into pBI121. Only selected restriction sites are indicated and the plasmids are not drawn to scale. Restriction sites: B, *Bam*HI; E, *Eco*RI; H, *Hinc*II; S, *Sac*I; Sm, *Sma*I; X, *Xba*I.

A 264 bp fragment was deleted from pSTN4, a cDNA clone containing the complete TNV CP coding gene (Fig. 3.9A). The plasmid was cut with *Eco*RI, made blunt with Klenow polymerase, cut with *Sma*I and religated. Deletion of the 264 bp fragment resulted in plasmid pCTZ1 (Fig. 3.9A.1). Recombinants with the correct deletion were confirmed by restriction analysis, and the sequence surrounding the newly engineered translation stop codons confirmed by sequence analysis. To confirm the translational integrity surrounding the end of the truncated CP gene it was subcloned into pMAL™-c (New England BioLabs) and shown to express in *E. coli* (Figs. 3.9A.2 & 3.10).

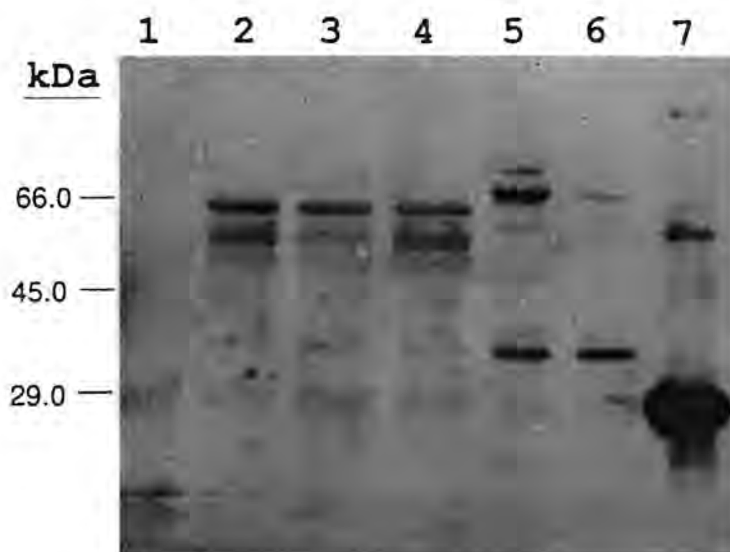

Fig. 3.10 Western blot analysis of the TNV-F5P full-length and truncated CP genes expressed in *E. coli* using anti-TNV IgG. Lane 1, cell extract of induced non-recombinant pMAL-c. Lane 2-4, induction of pMCC600 carrying the truncated TNV CP gene. Lane 5, induction of the full-length TNV gene from pMALc4 (section 4.3.3). Lane 6, uninduced pMCC600. Lane 7, purified TNV. Protein size markers are indicated on the left.

In order to facilitate directional and efficient cloning of the 638 bp truncated CP coding fragment into the plant expression vector pBI121, the blunt *Xba*I-*Hinc*II fragment from pCTZ1 was first cloned into the *Sma*I site of pUC19 (Fig. 3.9B). The presence and orientation of the insert was confirmed by restriction enzyme analysis. The insert in the correct orientation relative to the *Xba*I site of pUC19 resulted in plasmid pUITW. The *Xba*I-*Eco*RI (the latter site made blunt) TNV CP fragment from pUITW was subcloned into the *Xba*I-*Sma*I sites of pBI121, immediately downstream of the CaMV 35S promoter resulting in plasmid pBUX, with the truncated TNV CP sequence in the correct orientation with respect to the CaMV 35S promoter for expression of "sense" mRNA.

CHAPTER 4

Expression of the coat protein genes of CMV and TNV in *E. coli*

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CHAPTER 4

Expression of the coat protein genes of CMV and TNV in *E. coli.*

SUMMARY

Constructs containing both the full-length CP genes of CMV-Wem and TNV-F5P were subcloned in frame with the *malE* gene, which encodes the maltose binding protein (MBP), in the IPTG-inducible pMAL™ vector system, and expressed in *E. coli*. The highly-expressed 68K MBP::CMV CP and 74K MBP::TNV CP fusion proteins were immunoreactive with anti-CMV and anti-TNV polyclonal antiserum respectively in western blots. Antibodies prepared against the MBP::CMV CP fusion protein, were found to react both with purified CMV and with native CMV in plant sap.

4.1 INTRODUCTION

The coat protein genes of several plant viruses have been expressed in *E. coli*, usually in an effort to ensure that the gene was correctly identified, and that the cDNA clone expresses an appropriate protein (Nagel and Hiebert, 1985; Gal-On *et al.*, 1990; Brand *et al.*, 1993; Smith *et al.*, 1992). The endeavour to express the CMV and TNV CP genes in *E. coli* was undertaken for two reasons. The first was to provide a translation product from the cloned CP genes that could be used, through immunological verification, to confirm the authenticity of both genes. The second was to use the CP translation product expressed in *E. coli* as an antigen to raise antiserum free from contaminating plant host-specific antibodies, as antisera raised against biochemically purified plant viruses invariably contain antibodies against contaminating plant proteins.

The importance of having a polyclonal antiserum free from contaminating plant host-specific antibodies will become evident in Chapter 5, section 5.2.3. The coat proteins synthesized in transgenic plants have no assayable catalytic or enzymatic activities, and therefore anti-CP antibodies are the only means of detecting the potentially very dilute CP. This necessitates a high specific activity anti-CP antiserum which should have few or no antibodies which may react with

plant proteins with a similar molecular weight as the CP, thus potentially masking CP detection.

4.1.1 The pMAL™ series of vectors for the high-level expression of foreign genes in *E. coli*

The pMAL™ vector system (New England BioLabs; Fig. 4.1) provides a method for expressing and purifying large quantities of a protein expressed from a cloned gene or open reading frame (Guan, *et al.*, 1987; Maina, *et al.*, 1988). The gene of interest is inserted in-frame downstream of the *malE* gene which encodes the MBP, and results in the expression of a MBP fusion protein. The vectors contain the IPTG-inducible P_{tac} promoter positioned so as to transcribe a *malE-lacZα* gene fusion. The polylinker provides several restriction sites for insertion of the gene of interest between the *malE* and *lacZα* ORFs, which interrupts the *malE-lacZα* fusion, resulting in a change in colony colour from blue to white on X-gal in an α-complementing host. The expression from the P_{tac} promoter is kept low in the absence of IPTG by expression of the *lacI* gene, which codes for the *lac* repressor.

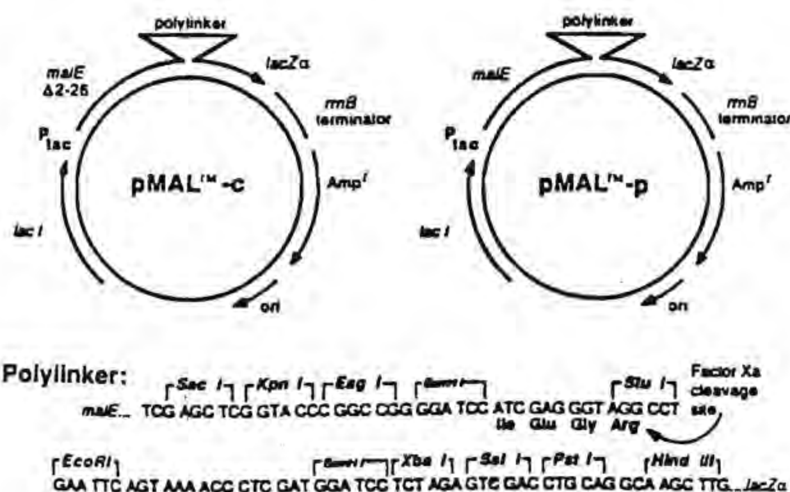

Fig. 4.1 pMAL™ *E. coli* expression vectors. The vectors contain the inducible P_{tac} promoter positioned to transcribe a *malE-lacZα* gene fusion. The expression from the P_{tac} promoter is kept low in the absence of IPTG by the expression of the *lacI* gene, which codes the *lac* repressor. The polylinker region is situated at the *malE-lacZα* fusion junction. The gene of interest can be fused in-frame to the *malE* gene, disrupting the *lacZα* complementation thus allowing blue-white selection. In order to cleave the protein product of interest the gene should be fused in-frame downstream of the protease (Factor Xa) cleavage site. pMAL™-c (6145 bp) has an exact deletion of the *malE* signal sequence. pMAL™-p (6220 bp) includes the *malE* signal sequence. (Figure taken from New England BioLab manual).

The main advantage of the pMAL™ vector system is that the MBP can be cleaved from the protein of interest. The vectors contain the sequence coding for the recognition site of the specific protease, factor Xa (Nagai and Thøgersen, 1987). Factor Xa cleaves after its four amino acid recognition sequence, as shown in Fig 4.1, so that few or no vector derived amino acid residues are attached to the protein of interest. For purification the crude cell extract is poured over an amylose affinity column. The MBP binds to the amylose, while all other proteins flow through. Pure fusion protein is then eluted using a buffer that contains maltose. The purified fusion protein can then be cleaved with the specific protease described above. The protein of interest is then separated from the MBP by passing the cleaved protein products over the amylose column. The MBP remains bound to the column while the cloned protein flows through.

For the purpose of this study, the vectors pMAL™-c and pMAL™-p were used: the unique difference between the two vectors is that in pMAL™-p the signal sequence of the *malE* gene is intact, potentially allowing fusion proteins to be secreted to the periplasm while, pMAL™-c has the signal sequence deleted, leading to cytoplasmic localization of expressed protein.

4.2 MATERIALS AND METHODS

Methods and techniques not described or referenced here were performed as described by Sambrook *et al.* (1989).

4.2.1 Expression of the MBP::CMV CP fusion protein in *E. coli*

Cultures of *E. coli* LK112 harbouring the recombinant pMAL™-c/p vectors were grown overnight at 37°C in LB plus 100 µg/ml Amp and diluted 1:250 in fresh medium. These were then grown for 2-3 h or until the culture reached a cell density of $2x10^8$ cells/ml (A_{600} = 0.4). A 1 ml sample was taken, microcentrifuged for 30 s, the supernatant discarded and the cells resuspended in 50 µl of SDS-PAGE sample buffer. IPTG was added to a final concentration of 0.3 mM to the remaining culture which was further incubated at 37°C for 2 h. A 0.5 ml sample was microcentrifuged for 30 s and the pellet resuspended in 100 µl sample buffer. All samples were boiled for 5 min and 15 µl of each sample electrophoresed on a 12.5% SDS-PAG.

For large scale purification of the MBP::CMV CP fusion, 800 ml of LB medium with Amp was inoculated with 5 ml of an overnight culture. This was grown at 37°C until the cells had reached an OD of approximately 0.4. The culture was induced through the addition of IPTG as above and grown at 37°C for a further 2 h, after which the cells were harvested at 4000 x g for 10 min and resuspended in 50 ml lysis buffer (10 mM Na_2HPO_4; 30 mM NaCl; 0.25% Tween 20; 10 mM EDTA, pH 8.0). The sample was frozen overnight, thawed in cold water and subjected to two rounds of French pressing (French pressure 1.1 x 10^5 KPa). The cell debris was removed by centrifugation at 9000 x g for 30 min. The crude extract was diluted 1:5 with column buffer (10 mM sodium phosphate pH 7.2; 0.5 M NaCl; 1mM EDTA) plus 0.25% Tween 20. This was then loaded onto a pre-swollen amylose affinity column (2.5 x 10cm) at a flow rate of 1 ml/min. The column was washed with 3 column volumes of column buffer plus 0.25% Tween 20, and then with 5 column volumes column buffer without Tween 20. The fusion protein was eluted with column buffer plus 10 mM maltose. The concentration of fusion protein was determined using the Bio-Rad protein assay (Bradford, 1976) and samples analysed by western blot (Towbin, 1979; Rybicki and von Wechmar, 1982).

4.2.2 Production and evaluation of CMV specific antiserum

Purified MBP::CMV CP (250 µl/injection) was emulsified with an equal volume of Freund's incomplete adjuvant for the immunisation of rabbits as described by Van Regenmortel (1982). Weekly injections of purified antigen were administered for four weeks, and four subsequent booster injections were given at monthly intervals. Serum was collected three weeks after the initial injections, and weekly thereafter. Polyclonal antiserum directed against the MBP::CMV CP fusion protein was host-absorbed twice with a 1:1 ratio of French-pressed (French pressure 1.1 x 10^5 KPa) *E. coli* extract and plant sap, before being evaluated by western blot.

4.3 RESULTS AND DISCUSSION

4.3.1 Expression of the CMV CP gene in *E. coli*

4.3.1.1 Subcloning of the CMV CP gene into the pMAL™ expression vectors

To verify the authenticity and confirm the translational integrity of the CMV-CP gene, the 817 bp *Sac*I-*Cla*I fragment was cloned into the *Eco*RI site of vectors pMAL™-c and pMAL™-p. In order to generate a MBP::CMV CP fusion gene with a single uninterrupted reading frame, a fragment containing the entire CMV CP ORF but lacking the two 5' stop codons had to be found amongst the deletion mutants generated by unidirectional exonuclease III shortening (see section 3.3.1). As shown in Chapter 3 Fig. 3.4A(1) there are two in-frame stop codons 45 and 57 nucleotides 5' of the favoured CMV CP ATG initiation codon. Deletion mutant plasmid DNA was initially subjected to restriction analysis, using pSCM9 as a marker, prior to sequencing. Two clones with putatively shorter fragments were selected and subjected to sequence analysis.

The 5' shortened clone, pSCM47, was used to isolate the CP for expression. The 817 bp *Sac*I-*Cla*I fragment was excised from pSCM47 and made blunt using Klenow polymerase. The blunt-ended fragment was separated from plasmid vector by agarose gel electrophoresis, and gel purified using the Geneclean[R] (Bio101, U.S.A.) method as described by the distributors. The altered CP fragment was ligated in frame into the blunt-ended and dephosphorylated *Eco*RI site of pMAL™-c and pMAL™-p (Fig. 4.2A). The recombinant plasmids were transformed into *E.coli* LK112. Positive recombinant clones were initially selected on the basis of their white colour on indicator plates. When the putative recombinants were transferred onto fresh LB Amp plates containing 80 µg/ml and 0.1 mM IPTG, the majority of white colonies did not grow at all well. This could be attributed to the overexpression of the foreign fusion protein: some fusion proteins are toxic to *E. coli* even at induction levels of 10 µM IPTG (New England BioLab Manual).

DNA extraction and restriction analysis of 12 growth defective colonies showed plasmids containing CP gene inserts in the correct orientation. *Sac*I cleaves 5' of the CMV CP gene in the pMAL™-c polylinker region, while *Eco*RI cleaves at insert position 672 from the 5' of the pSCM47 insert. This meant that a 726 bp

fragment would be released from recombinants containing the CP gene in the correct orientation, and a 187 bp fragment if the CP gene was in the incorrect orientation (Fig. 4.3). Insertion of the CMV CP gene fragment in the correct orientation resulted in plasmids pMALc40, 41 and 44.

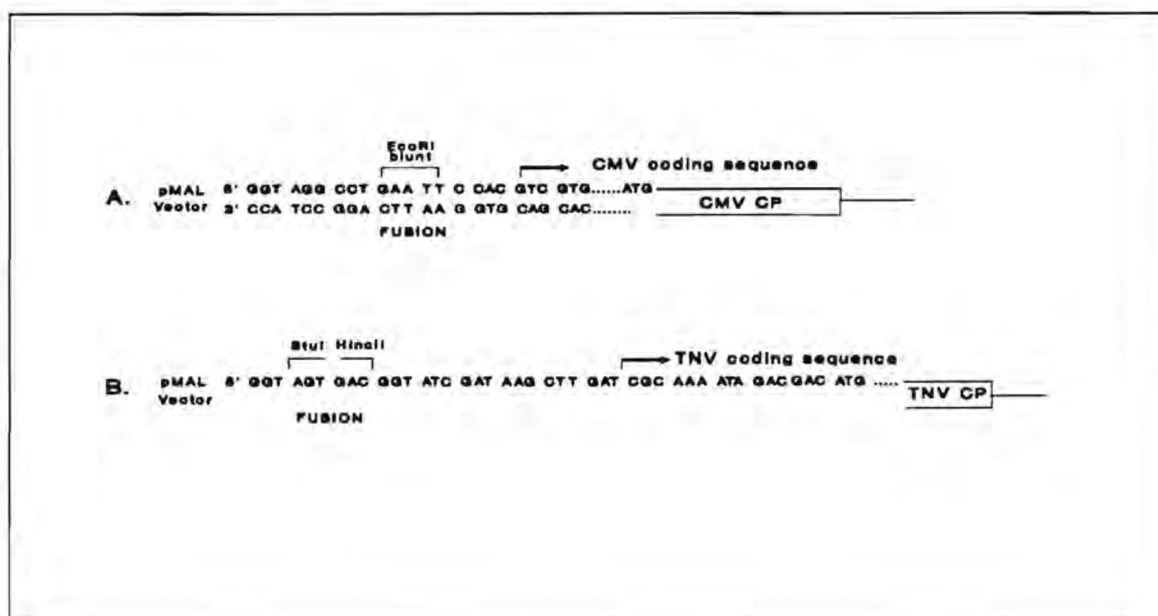

Fig. 4.2	Schematic representation of the CMV CP and TNV CP genes cloned into the pMAL^{TM} vectors.

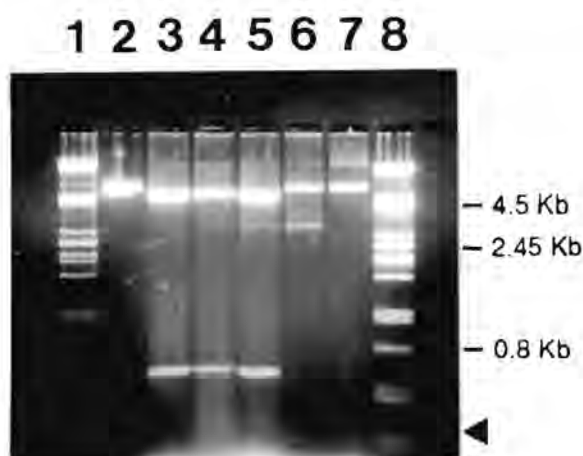

Fig. 4.3	Determination of the presence and orientation of the CMV-Wem and TNV-F5P CP gene inserts in the pMAL^{TM} *E. coli* expression vectors. Lanes 1 & 8, λ (*Pst*I). Lane 2, pMAL-c (*Eco*RI/*Sac*I). Lane 3, pMAL-c40 (*Eco*RI/*Sac*I). Lane 4, pMAL-c41 (*Eco*RI/*Sac*I). Lane 5, pMALc44 (*Eco*RI/*Sac*I). Lane 6, pMALc4 (*Eco*RI). Lane 7, pMALp5 (*Eco*RI). Arrow indicates the position of the 286 bp *Eco*RI fragment which are not easily visible in lanes 6 and 7.

4.3.1.2 SDS-PAGE and western blot analysis of the MBP::CMV CP fusion protein

The full-length protein expressed from a pMALc:CMV CP construct should contain an additional 15 amino acids at its N terminus, as a result of cloning part of the untranslated region 5' of the favoured start codon (Fig 4.2A). SDS-PAGE and western blot analysis of crude protein extracts from IPTG induced *E. coli* harbouring pMALc40, 41 or 44 confirmed that the MBP::CMV CP fusion was being synthesized. There was a single predominant product with a molecular weight (M_r) of 68 kDa (Fig. 4.4A), which is in good agreement with the predicted M_r of 68.1 kDa, taking into account the additional 15 amino acid residues. This is also about 26 kDa larger than the native MBP product, which has a M_r of 42 kDa. This served firstly as confirmation that the fusion protein being expressed was part CMV CP, and secondly as additional proof of the authenticity of the CMV CP gene. No serological reaction with CMV antiserum was observed for uninduced bacterial lysates or lysates containing pMAL-c (Fig. 4.4A).

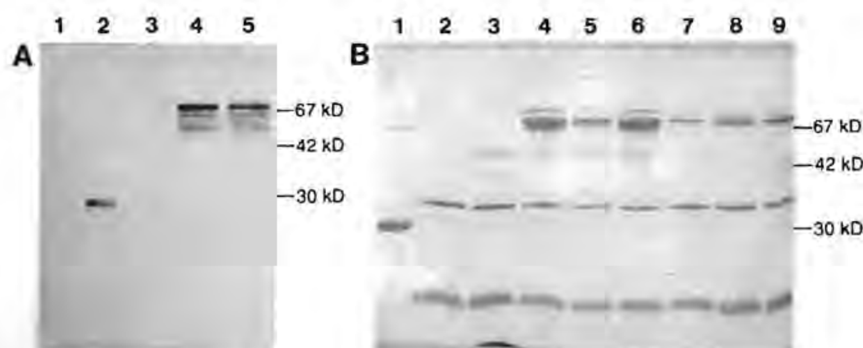

Fig. 4.4 Western blot analysis of the CMV CP and TNV CP expressed in *E. coli*. [A] Western blot of total *E. coli* protein cell extracts of clone pMALc41 probed with anti-CMV IgG. Lane 1, purified maltose binding protein (supplied by New England BioLabs). Lane 2, purified CMV. Lane 3, uninduced *E. coli* LK111 (pMALc41). Lane 4 & 5, LK111 (pMALc41) 1 h and 2 h after addition of IPTG respectively. [B] Western blot of total *E. coli* protein cell extracts of pMAL™ clones probed with anti-TNV IgG. Lane 1, purified TNV. Lane 2 & 3, uninduced and induced *E. coli* LK111 (pMAL-c) respectively. Lanes 4-9, recombinant clones, pMALc4, pMALc5, pMALc6, pMALp5, pMALp7 and pMALp8, induced with IPTG.

A number of extra bands were observed with the CMV antiserum in the size range between 51 kDa and the predominant 68 kDa MBP::CMV CP fusion protein. No reaction was observed for either the uninduced bacterial lysates or

lysates containing pMAL-c, suggesting that the bands are not non-specific unidentified bacterial proteins. These bands are therefore either the result of proteolytic degradation or were generated by premature termination of translation during gene expression. It is well known that proteolysis of heterologous proteins expressed in *E. coli* is a problem (Gold, 1990), although the rate of proteolysis is often slow enough to allow the accumulation of a high fraction of undegraded protein. This is clearly evident from both the SDS-PAGE (result not shown) and western blot results (Fig. 4.4A). In some cases, fusion proteins seem to be unstable, sometimes being degraded entirely and sometimes being degraded to the reporter protein moiety (Gottesman, 1990). Proteolysis may be the major reason for the extra bands, but mRNA secondary structures within the coding sequence can dramatically influence translation, and this cannot be ignored (Jagadish, *et al.*, 1991 & Schwartz, *et al.*, 1981).

4.3.1.3 Western blot with antiserum directed against the expressed MBP::CMV CP fusion protein

Western blot analysis showed that antiserum raised against amylose affinity column purified MBP::CMV CP fusion protein detected the CMV CP protein in purified preparations, as well as in the sap of CMV-infected plants (Fig. 4.5).

Fig. 4.5 Western blot analysis of purified CMV and CMV-infected plant material using polyclonal antiserum raised against the MBP::CMV CP fusion protein. Lane 1, purified CMV. Lane 2, purified TNV. Lane 3, healthy plant sap. Lane 4, sap from CMV-Wem infected plant. Lane 5, sap from CMV-Lupin K5 infected plant. Lane 6, sap from CMV-Q infected plant. Lane 7, sap from CMV-Y infected plant. Lane 8, sap from plant infected with an uncharacterised local isolate of CMV. Lane 9, *E. coli* extract of MBP::CMV CP fusion protein. Position of CMV CP band (MW 24,5 kDa) indicated with arrow.

4.3.2 Expression of the TNV CP gene in *E. coli*

4.3.2.1 Subcloning of the TNV CP gene into the pMAL™ expression vectors

For the same reasons mentioned in section 4.3.1.1, the gene encoding the putative full-length TNV CP was cloned into the pMAL™ *E. coli* expression vector system. The 890 bp fragment containing the TNV CP gene for expression in *E. coli* was subcloned from pTAG1, a cDNA clone containing the complete TNV CP coding gene (Chapter 3), into the pMAL™ expression vectors. pTAG1 was first cut with *Bam*HI and made blunt using the Klenow enzyme. The fragment was then released by cutting with *Hinc*II (a blunt cutter) and ligated into the dephosphorylated *Stu*I site of pMAL™-p and pMAL™-c (Fig.4.2B). The recombinant plasmids were transformed into *E. coli* JM105.

Twenty-four white colonies were chosen from the indicator plates, 12 clones with putative recombinant inserts into pMAL™-c, and 12 with inserts in pMAL™-p. Preliminary restriction analysis clearly showed plasmids with inserts. Further analysis was undertaken involving eight recombinants and these were subjected to *Eco*RI digestion.

The correct banding pattern and fragment sizes calculated for this digestion were seen by agarose gel electrophoresis, which confirmed the presence and orientation of the inserts. *Eco*RI cleaves twice, once in the TNV CP gene sequence at insert position 611 from the 5' end of the pTAG1 insert, and again in the polylinker sequence, 3' of the TNV CP insert. This meant that a single fragment would be excised from recombinants containing the CP gene. A 286 bp fragment (Fig. 4 3) if the insert was in the correct orientation, or a 646 bp and if the CP gene was in the incorrect orientation. Insertion of the fragment in the correct orientation resulted in plasmids pMALc4, c5, c6 and pMALp5, p7 and p8.

4.3.2.2 SDS-PAGE and western blot analysis of the MBP::TNV CP fusion protein

SDS-PAGE analysis of IPTG-induced *E. coli* JM105 carrying pMALc 4, 5, 6 and pMALp 5, 7, 8 revealed a large predominant protein band, which was not evident in the uninduced control (Fig. 4.4B). A M_r of 74 kDa was determined for the fusion protein which compares favourably to the predicted M_r of 73.2 kDa, about 30 kDa larger than the native MBP protein product. In *E. coli* the full-

length protein expressed from pMALc/p:CP constructs should contain an additional 11 amino acids at its N-terminus: 4 from the 5' region of the TNV CP, and the remaining seven from the multiple cloning site sequence from the pTAG1-derived insert (Fig. 4.2B).

The western blots confirmed that the fusion protein was in part TNV CP, and gave additional proof of the authenticity of the TNV CP gene expressed in *E. coli*. TNV antiserum did not react with a 74 kDa protein band in uninduced bacterial lysates or in lysates containing either pMAL-c or pMAL-p. However, extra bands similar to those observed in western blots using CMV antiserum were detected, as well as non-specific reactions with unidentified bacterial proteins. TNV-specific antiserum reacted with proteins smaller than the 74 kDa fusion from bacterial lysates of recombinant plasmids, but not from the controls. This may be attributed to proteolytic degradation or structural interference of translation, as discussed above for the MBP::CMV CP fusion protein.

It was not necessary to purify the MBP::TNV CP fusion in order to raise antisera against this protein as polyclonal antibodies raised against TNV virions were found to be suitable for western blot analysis of TNV CP+ transgenic plants (see Chapter 5). However polyclonal antibodies raised against CMV were found to be contaminated with antibodies raised against plant host components. For this reason we used the efficient pMAL system in order to express the CMV CP fusion protein, purify it and raise antiserum free from contaminating antibodies.

CHAPTER 5

Agrobacterium-mediated plant transformation and analysis of transgenic plants

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CHAPTER 5

Agrobacterium-mediated plant transformation and analysis of transgenic plants

SUMMARY

Transformation of *Nicotiana tabacum* cv Petit Havana SR1 leaf discs with a variety of plasmid constructs, resulted in a large number of transgenic plant lines. These plants were subsequently analysed for the presence and expression, at the transcriptional and translational level, of both the CMV-Wem and TNV-F5P CP genes in sense and antisense orientations. Upon mechanical inoculation of R_o lines with CMV-Wem more than 50% of the CMV CP+ and CP-antisense plants did not develop visible systemic disease symptoms. Virus accumulation was delayed in the CMV CP+ transgenic plants, but virus did accumulate in the inoculated leaves over time. The CMV CP+ lines showed excellent protection against CMV-Q, a strain belonging to the homologous CMV subgroup, but only showed a delay in systemic symptom development when inoculated with CMV-Y (heterologous subgroup). The results obtained suggest that there is possibly more than one mechanism involved in the resistance observed. These mechanisms may be in operation at the initial stage of virus infection (early event) and at a later stage during virus movement (later event).

Mechanically inoculated transgenic plants expressing either the full-length TNV-CP, the CP antisense transcript or a truncated form of the CP, showed a reduction in necrotic lesions. It is unclear whether just the truncated form of the TNV CP or specifically the N-terminus is required for the resistance observed. However it appears that expressing a dysfunctional CP in transgenic plants is an effective means of mediating virus resistance.

5.1 INTRODUCTION

5.1.1 Natural *Agrobacterium tumefaciens* mediated gene transfer in plants

A. tumefaciens infection of wounded dicots results in proliferative tumorous growths called crown galls. This pathogenic soil bacterium harbours a large plasmid, designated the Ti (for tumour-inducing) plasmid, which is the cause of the bacterium's oncogenic capabilities (Van Larebeke *et al.*, 1974). Tumour formation is initiated by the transfer of part of the Ti plasmid DNA (T-DNA) from the bacterial cells to the infected cells and integration into the nuclear genome (Chilton *et al.*, 1980; Zambryski, 1988).

The genes that are encoded by the T-DNA are functional in plant cells. Oncogenic genes encode enzymes for the biosynthesis of auxin and cytokinin which disrupt the normal hormonal balance of the cell resulting in the tumorous phenotype (Walden and Schell, 1990). In addition to these genes the T-DNA also encodes enzymes responsible for the production of specific opines, which are either condensates of an amino acid and a sugar (e.g. octopine, nopaline, leucinopine, agropine) or phosphorylated sugar derivatives (agropines). Virtually all virulent *A. tumefaciens* strains catabolize the specific opines produced by the tumours they induce.

The T-DNA region of the Ti plasmid is the only fragment to be transmitted from the bacterium to the plant cell. None of the T-DNA encoded genes are essential for transfer of this region into plants. The ss-DNA molecule called the T-strand must be bordered by a set of 25 base pair "integration sequences", the left and right borders, in order for transfer to take place. This process is thought to be analogous to the conjugal transfer mechanism of bacterial plasmids. *Agrobacterium* cells that harbour the necessary virulence (*vir*) genes can transfer any DNA segment, whether it has a plasmid or chromosomal location, provided it is bordered by the integration sequences.

5.1.2 T-DNA transfer and the *vir* regulon

The *vir* region of the Ti plasmid encodes proteins which sense and respond to the presence of phenolic compounds, such as acetosyringone and hydroxyacetosyringone, released by wounded plant cells (Bolton *et al.*, 1986).

These Vir proteins, which operate in *trans*, are responsible for the appearance of the ss-DNA T-strand and its transfer to the plant cell.

The following is a brief summary of the cascade of interactive events which take place from the time the *Agrobacterium* senses the wound induced chemical signal until the T-DNA integrates into the plant genome (for other author reviews see Ream, 1989; Hooykaas, 1989).

There are 22 virulence genes located on the octopine type Ti plasmid present in 7 operons called *virA-virG*. These operons are co-regulated and thus form a regulon. The protein products VirA and VirG mediate the activation of the other *vir* genes in the presence of phenolic inducers. After VirA has come into contact with acetosyringone it is able to activate (phosphorylate) the VirG polypeptide, which is located in the bacterial cytosol. The phosphorylated VirG polypeptide acts as a transcriptional activator of all the other *vir* genes (Zambryski, 1992).

The gene products of the *virB* operon are absolutely essential for T-DNA transfer, and according to the DNA sequence might determine a physical structure (a pore or pilus) through the bacterial envelope, making transfer possible. The first two genes of the *virD* operon encode an endonuclease activity that generates specific single-stranded nicks and double-strand cuts within the border repeats. The bottom strand is liberated from the parental plasmid, and VirD2 protein attaches covalently to the 5' end of the nicked DNA. The "overdrive" enhancer (*ode*) sequence is located next to the right border repeat and stimulates both tumour induction and production of T-strands, apparently with VirD2 protein and with one or both of the VirC proteins. A nonspecific ssDNA binding protein (SSB) encoded by *virE2* binds and protects the T-strands, while VirE1 protein may increase the stability of the VirE2 protein. The attachment of VirD2 to the 5' end of T-strands probably allows the protein to have added functions, such as guiding the T-strands to the nucleus of the recipient (plant) cells and aiding the T-strand integration into the plant nuclear DNA by cutting the target DNA. Thus, complex DNA-protein interactions govern transmission of T-DNA to plant cells (Zambryski, 1992).

For plant genetic manipulation, modified or "disarmed" *A. tumefaciens* strains are used in which the tumour inducing genes have been removed from the T-DNA and replaced by the genes to be transferred.

5.2 MATERIALS AND METHODS

Methods and techniques not described or referenced here were performed as described by Sambrook *et al.*, 1989.

5.2.1 Transfer of the Ti vectors containing the CMV and TNV CP genes into *A. tumefaciens*

Mobilization of the sense and antisense chimaeric CP plasmid constructs into *Agrobacterium* was carried out either by the triparental mating method of Deblaere *et al.* (1987), or the direct *Agrobacterium* transformation method of Holsters *et al.* (1978). Triparental transconjugants were verified as *Agrobacterium* using the 3-ketolactose test described by Bernaerts and De Ley (1963). *Agrobacterium tumefaciens* produce 3-ketoglycosides which reduce the sugar reagent Benedict's, resulting in a yellow ring visible around the cell mass. Integration of the cointegrate vector constructs was confirmed by total *Agrobacterium* DNA isolation using the method described by Dhaese *et al.* (1979) and by Southern hybridization. Probes used were nick-translated ^{32}P-labeled DNA prepared from gel-purified fragments of cDNA containing the CP ORF.

Confirmation of binary vector transformation in *Agrobacterium* was carried out by an alkali lysis quick-screen procedure (Webster and Thomson, 1988). 1 ml of an overnight culture was centrifuged and the pellet resuspended in 150 µl E buffer (40 mM Tris acetate, 2 mM EDTA, pH 7.9). 300 µl lysing solution (3% SDS, 50 mM Tris, pH 12.6 with NaOH) was added, mixed gently and incubated for 2.5 min at 95°C, followed by addition of 40 µl 1 M TE and incubation on ice for 5 min. 900 µl phenol:chloroform:iso-amylalcohol (25:24:1) was added, the contents mixed gently and centrifuged for 15 min at 4°C. This was left at 4°C for 15 min and then 50µl of the aqueous phase separated in a 0.7% agarose gel in E buffer without ethidium bromide. Southern hybridization using a digoxigenin (DIG)-labeled probe prepared by the polymerase chain reaction (Lanzillo, 1990), and subsequent chemiluminescent detection (Boehringer Mannheim) confirmed the presence of the insert

5.2.2 Plant transformation

Leaf discs of *Nicotiana tabacum* cv Petit Havana line SR1, or a transformant of this parental strain, were transformed by the method of De Block *et al.* (1987), with some modifications. *Agrobacterium* strains transformed with the desired construct were cultured in LB medium with antibiotic selection. Cell

suspensions were pelletted at 5000 rpm for 5 min, and the cells resuspended in 10 mM $MgSO_4$. This was repeated and cells resuspended to give a final concentration of 10^6-10^8 c.f.u/ml, where 1 OD_{600} = $8x10^8$ c.f.u./ml. Fresh explants were incubated in Petri dishes, lower epidermis down, for 20 min on a slow shaker. Explants were removed and co-cultivated with the bacteria on plant medium, $^1/_2$ MS salts (Murashige and Skoog, 1962), 3% sucrose, 0.1 µg/ml naphthalene acetic acid (NAA), 1 µg/ml benzylamino purine (BA), 0.8% gel (Sigma)(pH 5.8 with KOH), without antibiotic selection for 2 days in the dark, with the lower epidermis up. The explants were then subcultured onto the same medium containing cefotaxime (250 µg/ml), Km (300 µg/ml) and/or hygromycin (20 µg/ml) (Sigma) for selection. In order to limit callus production and stimulate shoot formation directly from the infection site, NAA and BA were used at the above final concentrations, in the medium. Morphologically normal shoots were excised and placed on $^1/_2$ MS medium without NAA and BA, but with antibiotic selection.

Plants which rooted under antibiotic selection were transferred into a 1:1 bark and polystyrene mix and sealed under plastic to maintain a moist environment for a 7 day hardening-off period in the greenhouse. Maintenance conditions in the greenhouse were 25°C and 16 hours light. The plants were then transferred to larger pots containing potting soil. A clone of each plant was maintained in axenic culture on $^1/_2$ MS medium, 3% sucrose, cefotaxime (250 µg/ml), Km (100 µg/ml) and/or hygromycin (20 µg/ml).

5.2.3 Identification of transgenic R_0 tobacco plants: northern and western blot analysis

Northern blot analysis. Total plant RNA was isolated using the rapid small scale procedure of Verwoerd *et al.* (1989). Approximately 10 µg of RNA was separated in a 1% agarose gel under non-denaturing conditions, with no formaldehyde in the gel. The RNA was then transferred to a nylon membrane (Hybond N+, Amersham) via a dry alkali blotting procedure as recommended by the manufacturer. Prehybridization and hybridization conditions were as described for the DIG detection procedure (Boehringer Mannheim). For the analysis of CMV CP transgenic plants strand-specific riboprobes were prepared using T3 or T7 DNA-dependent RNA polymerase reactions with pSCM47 linearized with the restriction enzymes *Hin*dIII or *Sac*I, respectively. Similarly for the analysis of TNV CP transgenic plants the plasmid pTAG1, linearised with

*Cla*I and *Bam*HI, was used to generate T3 and T7 riboprobes, respectively. In all cases the transcript probes were labeled with the DIG hapten (DIG-11-dUTP) (Höltke and Kessler, 1990) and the membrane-bound probe immunologically detected using anti-DIG Ab conjugate to alkaline phosphatase, Lumigen-PPD™ (Boehringer Mannheim) as the enzyme substrate, as recommended by the manufacturer.

Western blot analysis. Between 100-200 mg of leaf tissue were ground in 400 µl of 2x Laemmli running buffer (1x Laemmli buffer: 25mM Tris; 192mM Glycine; 0.1% SDS pH 8.3; Laemmli, 1970) in 1.5 ml tubes, and clarified by centrifugation in a microcentrifuge for 10 min at 10 000x*g*. Protein concentration was estimated by the dye-binding procedure of Bradford (1976) (Bio-Rad Protein Assay) using bovine serum albumin (BSA) as a standard. Protein samples (50 µg of total protein) were separated on a 12.5% SDS polyacrylamide gel and immunoblotted onto nitrocellulose (Schleicher and Schuell Inc, BA 85) as described by Towbin *et al.* (1979). Western blots were performed by the method of Rybicki and von Wechmar (1982). Blots were developed using anti-CMV CP and anti-TNV CP as the primary polyclonal antibodies, alkaline phosphatase-conjugated goat anti rabbit (GAR) secondary antibodies (Sigma) and the Lumigen-PPD™ chemiluminescent substrate (Boehringer Mannheim) to visualise the CP. Nitrocellulose membranes were treated with Nitro-block™ reagent (Tropix) prior to substrate incubation as recommended by the manufacturer.

5.2.4 Inoculation of transgenic plants

The viruses used were CMV-Wem, CMV-Q (subgroup II), CMV-Y (subgroup I) and TNV-F5P (kindly donated by M.B. von Wechmar, UCT collection).

CMV infectivity assays. R_0 plants which were approximately 15 cm tall were inoculated with CMV-infected plant sap. The inoculum was mixed with Celite and applied mechanically with a cottonwool swab to the lowest two leaves of the plant. The infected plant sap inoculum was prepared by grinding CMV-infected *N. glutinosa* leaf tissue (2 wk post inoculation) in ice-cold 0.2 M sodium phosphate buffer (pH 7.5) containing 0.1% thioglycollic acid, at a ratio of 1 g:10 ml. Inoculated plants were examined daily, and the appearance and/or severity of systemic symptoms were recorded, generally up to 18-30 days post-inoculation. Symptoms on any leaf above the inoculated leaves were considered systemic.

TNV infectivity assays. Leaves of taller (approximately 30 cm) plants were removed from the plant and inoculated with 100 ul of the appropriate dilution of purified TNV virions. This dilution gave about 100 lesions on control *N. tabacum* non-transgenic leaves. The TNV virions were purified as described in section 2.2.1. Celite was mixed with the inoculum and the entire leaf surface mechanically inoculated. Leaves were placed in a sealed plastic bag to maintain a moist environment and incubated at RT. Inoculated leaves were scored for the number of local necrotic lesions 3 to 7 days after inoculation.

Protein dot blot analysis. Eppendorf lid size disks were sampled from inoculated plants. Leaf tissue was ground in phosphate buffered saline (PBS), pH 7.3 to 7.4, in a ratio of 2 ml buffer/g fresh weight of tissue. The extracts were then clarified by centrifugation (10 min at 10,000x*g*) and the protein was quantitated in the supernatants as described by Bradford (1976) (Bio-Rad Protein Assay). Protein samples (10 µg of total protein) were applied to nitrocellulose using a suction blot apparatus (Hoefer Scientific Instruments). Prior to applying the samples the nitrocellulose was soaked in PBS. After applying the last sample the membrane was baked for 15 min at 65°C and then rinsed in wash buffer (PBS, 0.1% Tween 20). The membrane was blocked by incubating in wash buffer containing 5% milk powder (Oxoid) and all other procedures followed as described for western blot analysis.

5.3 RESULTS

5.3.1 Plasmid transfer into *A. tumefaciens*

Construction of the chimaeric CP-carrying plant vectors is described in detail in Chapter 3 and summarised diagrammatically (Fig 5.1). This figure also shows the *A. tumefaciens* strains and associated disarmed Ti plasmids used for each construct. Plasmids pGSC315, pGAC340 and the cointegrate vector pGSJ280 the latter containing no inserted sequences were introduced into the *A. tumefaciens* strain C58C1 (pGV2260) (Deblaere *et al.*, 1985) by the triparental mating procedure using pRK2013 as a helper plasmid (Figurski and Helinski, 1979). Southern blot hybridization of total *Agrobacterium* DNA from putative transconjugants with a CMV-Wem specific probe confirmed the presence of the CP gene.

Construct	Vector	*Agrobacterium* Strain(s)	Transgenic Line(s)	# Plants
P35 — SENSE CMV-CP (↑↑↑)	pGSJ280	C58C1(pGV2260)	GSC318	6
P35 — ANTISENSE CMV-CP	pGSJ280 pGA643	C58C1(pGV2260) C58C1(pMP90)	GAC340 GCMA	18 21
P35 — SENSE CMV-CP	pGA643	C58C1(pMP90)	GCMS	40+
P35 — SENSE TNV-CP	pGA643 pBI121	C58C1(pMP90) LBA4404(pAL4404)	GAT BITS	21 25
P35 — ANTISENSE TNV-CP	pBI121	LBA4404(pAL4404) C58C1(pMP90)	BITA	27+
P35 — TNV-CP△ (⇧⇧)	pBI121	LBA4404(pAL4404) C58C1(pMP90)	BUX	15
P35 — SENSE TNV-CP (HYG-R)	pGA643	C58C1(pMP90)	CMTN	19

Fig. 5.1 Forms of the CMV-Wem and TNV-F5P CP genes inserted into *Nicotiana tabacum* cv Petit Havana (SR1). All constructs contained the CaMV 35S promoter (P35) and a polyadenylation (transcription) terminator sequence. The resulting product in the transgenic plants is presented in the large open box of each representative construct. The lined box represents the 5' or 3' untranslated region. Filled-in arrows show the position of the three ATG codons. Open arrows indicate the position of the two stop codons created as a result of the construction of this truncated form. # Plants: this is the number of transgenic plants derived from the transformation of a particular form, kept in axenic medium.

Plasmids pGCMA and pGCMS were introduced by transformation into *A. tumefaciens* C58C1 (pMP90) (Koncz and Schell, 1986). The TNV CP chimaeric constructs were introduced in the same way into either C58C1 (pMP90) or into the Ach5 strain LBA4404 (pAL4404) (Hoekema *et al.*, 1983). Putative *Agrobacterium* transformants of the above binary vector chimaeric CP constructs and control pGA643 and pBI121 plasmids, containing no inserted sequences, were analysed by the alkali lysis quick-screen procedure (results not shown). The direct transformation method was found to be a reliable and rapid way of introducing the desired chimaeric binary vector constructs into *Agrobacterium*.

5.3.2 Plant regeneration and selection

Transformation of between fifty and one hundred *N. tabacum* cv Petit Havana line SR1 virus-free leaf discs with each recombinant *A. tumefaciens* strain, resulted in large numbers of discs producing shoots. After selection on root inducing medium a high proportion (80-90%) of the shoots formed roots. However the shoots transformed with the TNV CP antisense construct (pBITA45) and the CMV CP-transgenic shoots transformed with the TNV CP construct (pGAT12-H4) were different. In the case of the pBITA45 transformants, young shoots were morphologically normal, but when they were transferred to rooting medium a lower percentage rooted. There was also a significant delay in the rooting of a number of plants and these became morphologically abnormal. The stems elongated, with large internodal distances, and in some cases early flowering occurred. In the second case an extremely small percentage of the pGAT12-H4 transformants rooted. The MIC of hygromycin on untransformed *N. tabacum* was found to be 20 µg/ml. Callus growth could be selected on a hygromycin concentration of 100 µg/ml when leaf discs were transformed with the pGAT-H4 construct. After a number of attempts it was decided to select for shoot generation on a higher hygromycin concentration (50-100 µg/ml) than the mimimum inhibitory concentration (MIC) value, and then to transfer these shoots to rooting medium containing no hygromycin.

The number of plant transformants resulting from each CP construct is shown in Fig. 5.1. Plants showing no apparent abnormalities were selected for further analysis.

5.3.3 Identification of R_0 transgenic plants

It was important to determine if the putative transgenic SR1 tobacco plants produced the expected RNA and protein products. Total RNA samples isolated from the various transgenic lines were analyzed in northern blot hybridization studies. An RNA transcript of approximately 850 nt was expected for all CMV CP transgenic lines. Such a CMV CP transcript was detected in CP-antisense expressing plant lines by using a "plus"-sense riboprobe containing the CMV CP sequence (Fig. 5.2 A). An RNA transcript of approximately 1,200 nt was expected for all TNV CP plant lines expressing the full-length CP sequence in either orientation. The appropriate TNV CP transcripts were detected with both "minus"- and "plus"-sense TNV CP sequence riboprobes (Fig. 5.2 B,C). The

predicted RNA transcript size of 950 nt was confirmed for the truncated TNV CP expressing transgenic plant lines (Fig. 5.2 D). The expected RNA transcripts were detected in plants transformed with both the CMV and TNV CPs using the TNV minus-sense CP sequence riboprobes described above (Fig. 5.2 E). In all cases differing levels of CP transcript accumulation was observed in the different transgenic plant lines.

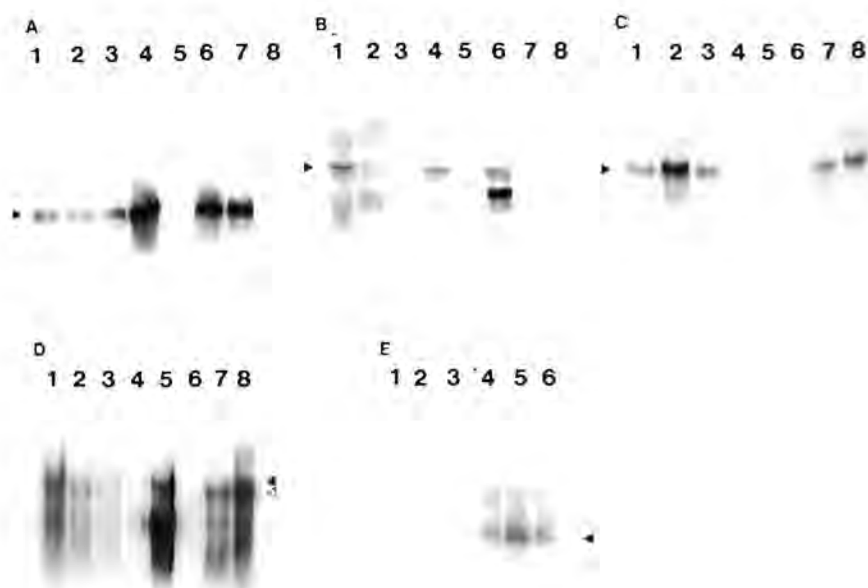

Fig. 5.2 Northern blot analysis of total RNA extracted from transgenic plants. Samples (10 µg) of RNA from R_0 transgenic plants were separated in a 1% agarose gel without formaldehyde. RNAs were transferred to nylon membranes (Hybond N+, Amersham) and hybridized with either plus- or minus-sense DIG-labelled riboprobes (Boehringer Mannheim). RNA present in each lane is as follows: [A] Analysis of transgenic plants expressing the CMV CP-antisense RNA transcript. Lanes 1, positive control T7 RNA polymerase transcription of pSCM47; 2, RNA sample from leaf tissue of transgenic plant lines GCMA-1; 3, GCMA-2; 4, GCMA-3; 5, non-transformed SR1; 6, GCMA-4; 7, GCMA-5; 8, RNA sample from plants transformed with pGA643 vector only. [B] Analysis of transgenic plants expressing the TNV CP+ RNA transcript. Lanes 1, positive control T7 RNA polymerase transcription of pTAG1; 2, GAT2. 3, GAT3; 4, vector pGA643; 5, GAT4; 6, GAT5; 7, GAT6; 8, GAT8. [C] Analysis of transgenic plants expressing the TNV CP-antisense RNA transcript. Lanes 1, BITA1; 2, BITA2; 3, BITA3; 4, BITA4; 5, vector pGA643; 6, BITA5; 7, BITA9; 8, GAT8 control. [D] Analysis of transgenic plants expressing the truncated form of the TNV CP (CPΔ). Lanes 1, BUX1; 2, BUX2; 3, BUX3; 4, BUX4; 5, GAT2 control; 6, non-expressor SR1; 7, BUX8; 8, BUX10. Closed arrow indicates position of full-length TNV CP RNA transcript. Open arrow indicates position of the truncated TNV CP RNA transcript. [E] Analysis of the doubly transformed transgenic plants expressing both the CMV and TNV CPs with a minus-sense TNV CP riboprobe. Lanes 1, BITA1 control; 2, CMTM 4.1; 3, non-expressor SR1; 4, CMTM 4.5; 5, CMTM 7.1; 6, CMTM 7.2.

Transgenic plant lines expressing full-length CMV-Wem and TNV-F5P CP were identified by western blot analysis (Fig. 5.3 A,B). The various CP products produced in the plants were stable and accumulated to different levels in individual transgenic plant lines. The two full-length forms could be readily distinguished from the bands resulting from the non-specific antiserum reactions.

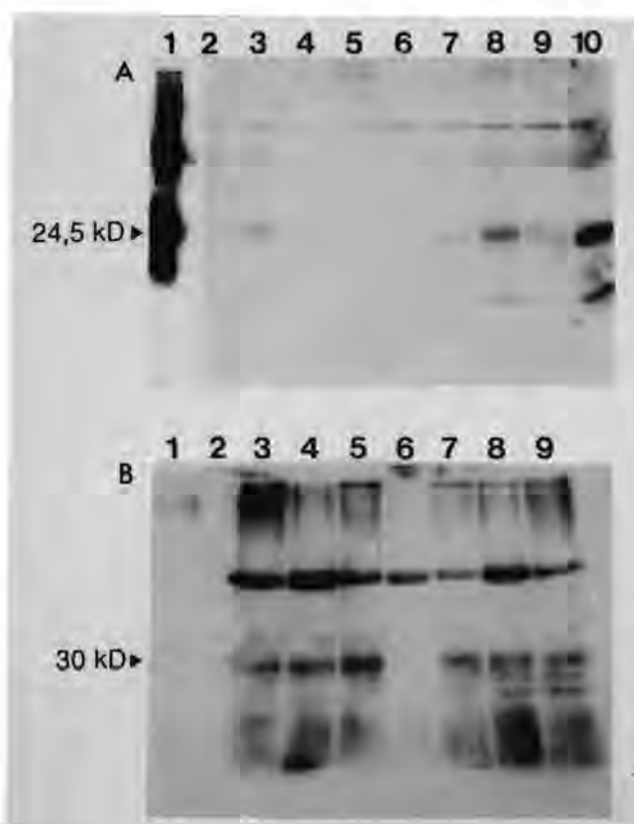

Fig. 5.3 Western blot analysis of transgenic plants. Protein samples (50 µg per lane) from leaf tissue of transgenic plants were separated on a 12.5% SDS-polyacrylamide gel and transferred to nitrocellulose. Bound antigen was detected with rabbit anti-CMV CP and anti-TNV CP polyclonal antisera, goat anti-rabbit (alkaline phosphatase conjuate) antibody, and the chemiluminescent substrate Lumigen-PPD™ (Boehringer Mannheim). [A] Analysis of CMV-Wem CP+ transgenic plants. Lanes 1, CMV-Wem positive control; 2, non-transformed SR1 negative control; 3-10, contain protien samples from GCMS transgenic plant lines, numbers 1-8 (GCMS-1 - GCMS-8). [B] Analysis of TNV-F5P CP+ transgenic plants. Lanes 1, non-transformed SR1 negative control; 2, TNV-F5P positive control; 3-10, contain protein samples from GAT transgenic plant lines, numbers 1-8 (GAT1 - GAT8). CMV and TNV CP sizes are shown on the sides of the figures.

5.3.4 Protection of R_o plants against mechanical inoculation

In order to estimate the level of virus resistance in transgenic tobacco plant lines expressing forms of the CMV and TNV CP genes, plants were challenged with

CMV or TNV. In the case of the doubly transgenic lines, plants were challenged with both viruses.

Susceptibility of transgenic plants to primary virus infection by CMV virions. Inoculation of SR1 plants with CMV-Wem (plant sap) typically resulted in mosaic symptoms on systemically infected leaves approximately 5-7 days after inoculation. Sixteen randomly selected CMV CP expressing R_0 transgenic plants (GCMS-line) and twenty-one CMV CP antisense expressing transgenic plants (GCMA-line) were mechanically inoculated with a 1:15 homogenate prepared from the upper leaves of a CMV-Wem infected plant. As controls non-transformed SR1 plants were challenged with the same inoculum. Following inoculation visual symptoms could be seen on the sytemically infected leaves within 6-7 days post inoculation (d.p.i.) for the non-transformed plants and some transformed plants. However more than 50% of both sense and antisense transgenic plants did not develop disease symptoms (Fig. 5.4 A). Line GCMS-3 (CP+) was highly resistant and showed no systemic symptom development by 30 d.p.i. In contrast line GCM-8, which expressed significant quantities of CMV CP, showed very little reduced degree of resistance with marked systemic symptoms. This is more fully discussed on P104.

CMV CP sense protection against CMV-Q (subgroup II) and CMV-Y (subgroup I). Quemada *et al.* (1991) reported that transgenic tobacco plants that expressed the CP gene from CMV-C (subgroup I) were well protected from infections by virus strains from the homologous subgroup. The same plants also appeared to be protected from infection by a strain from the heterologous subgroup, CMV-WL (subgroup II) although the level of protection was not as pronounced. The CP sequence of CMV-Wem has 98% amino acid sequence identity with the sequences of CMV strains from subgroup II, and as low as 76% amino acid sequence identity with strains in subgroup I. Transgenic tobacco plants (GCMS-lines), expressing the CMV-Wem CP gene were vegetatively propagated and inoculated with either CMV-Q or CMV-Y in order to investigate the range of CP-mediated resistance. The results (summarized in Fig. 5.4 B) show that greater than 90% of the 24 GCMS-CP(+) plants do not exhibit systemic mosaic symptoms up to 30 d.p.i., when inoculated with plant sap containing CMV-Q virions (same subgroup). Most of the the non-transformed control plants developed systemic disease symptoms within 7-10 d.p.i. Systemically infected plants also displayed severe stunting and leaf deformation.

When sap containing CMV-Y (heterologous subgroup) was inoculated onto 21 GCMS-CP(+) plants and control plants, disease symptoms developed on the inoculated leaves of the control plants within 5 days and in systemic leaves after 7 d.p.i. The CP(+) transgenic plants showed a significant level of resistance until at least 11-12 d.p.i. (Fig. 5.4 C). At 14 d.p.i. four transgenic plants were completely symptomless (Fig. 5.5 A), with the same number of plants showing the beginnings of systemic symptoms. Three different stages of CMV-Y infection were noted as shown in Fig. 5.5 B. Each inoculated plant was scored for the leaf number from the inoculated leaf which showed the first stages of infection (Table 5.1). These results indicate that disease development in the transgenic plants is not manifested in the same way as in the control non-transformed plants, and indicates some degree of resistance against symptom development.

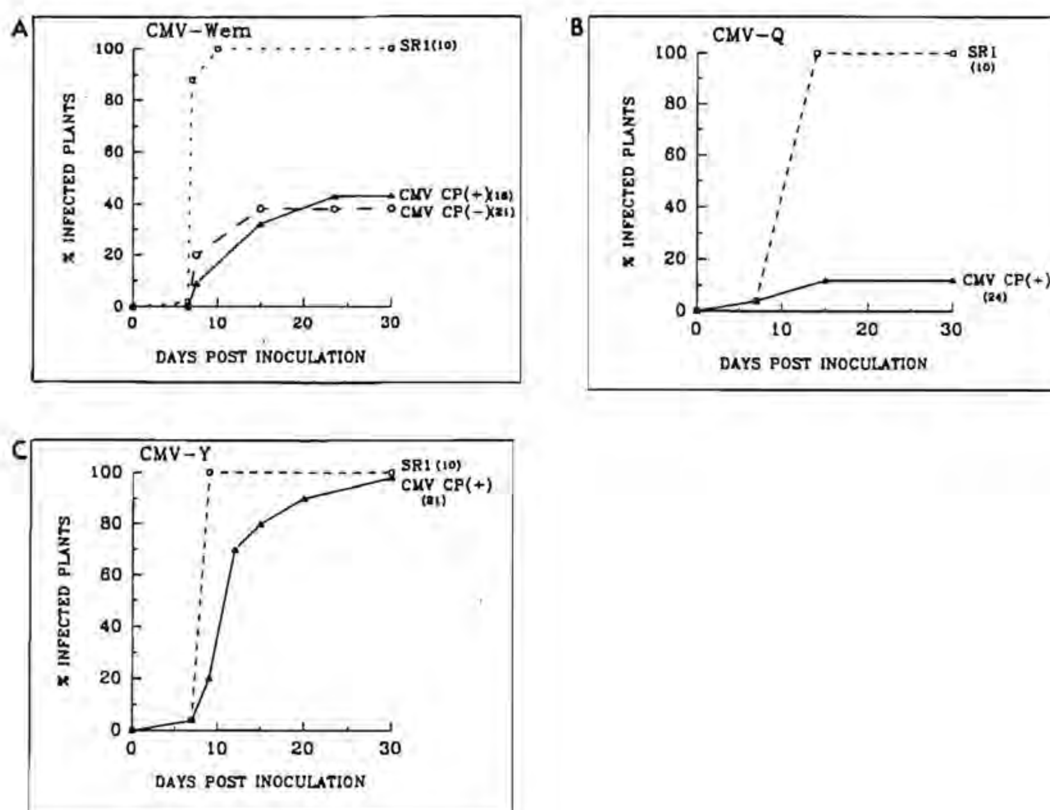

Fig. 5.4 Development of systemic symptoms in control and transgenic plants after inoculation with CMV. Non-transformed SR1 (wild type) plants and R_0 plants of transgenic plant lines were mechanically inoculated with 100 µl of 1:15 dilution of CMV infected plant sap. In all experiments ten SR1 plants were inoculated with the respective CMV as positive controls [A] 16 CMV CP+ (GCMS)and 21 CMV CP-antisense (GCMA) transgenic plant lines inoculated with CMV-Wem. [B] 24 GCMS transgenic plant lines inoculated with CMV-Q. [C] 21 GCMS transgenic plant lines inoculated with CMV-Y. Plants were examined daily for the appearance of systemic symptoms and any plants displaying systemic symptoms (attenuated or wild type) were recorded as symptomatic.

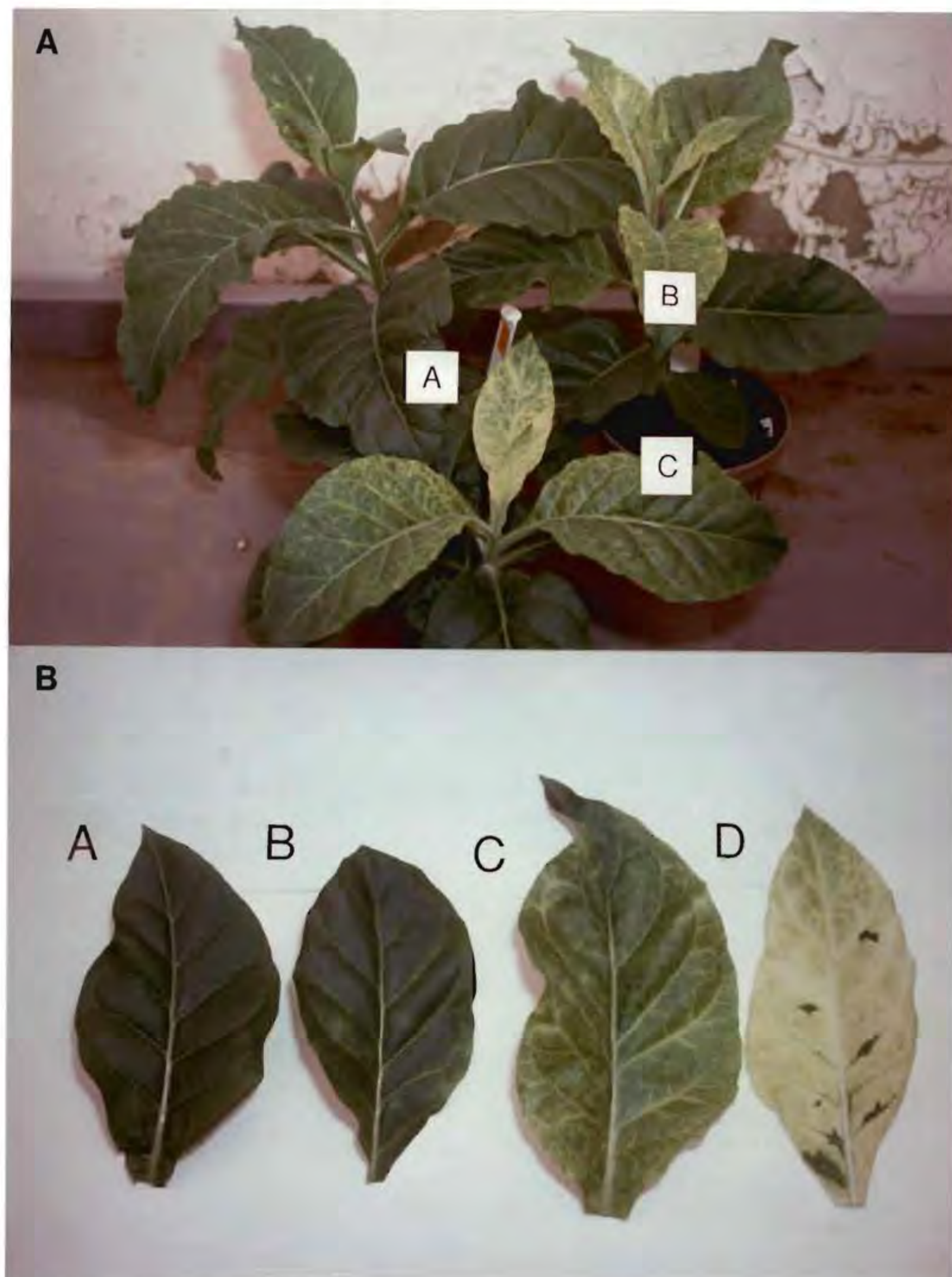

Fig. 5.5 [A] Comparison of symptom development in non-transformed SR1 plants (C) and GCMS CMV CP+ (A & B) transgenic plants after inoculation with CMV-Y. The plants were photographed three weeks after inoculation with CMV-Y. [B] Comparison of the stages of wild type CMV-Y systemic infection in the leaves of non-transformed SR1 plants. The CMV-Y infection can be manifest from low (B) to severe (D) systemic disease development. This depends on the leaf number above the inoculated leaf and the number of days post inoculation (d.p.i.). Leaf (A) is a symptomless control. Samples were taken 8 d.p.i.

Table 5.1 Comparison of resistance to CMV-Y induced by CMV-Wem CP transformation of *Nicotiana tabacum*.[1]

	Leaf number above the inoculated leaf[2].		
Transgenic line	*Stage 1*[3]	*Stage 2*	*Stage 3*
SR1 control	1	3	6
SR1 control	1	2/3	5
GCMS-8	3	4/5	6
12	2	5	7/8
6	5	6/7	8
3	3/4	6	8
20	5/6	7/8	10
5	4	5/6	7
10	5	6/7	8
19	5/6	8	
17	nil		
21	nil		
26	nil		

1 Results taken 20 d.p.i.
2 Leaf number above the inoculated leaf showing a stage of symptom development.
3 Three stages of symptom development as seen in Fig. 5.5B.

Protein dot-blot analysis of virus accumulation in CMV CP+ plants following inoculation with CMV-Wem. Inoculation with the CMV-Wem isolate resulted in an overall delay in systemic symptom development in CMV CP+ transgenic plants. Furthermore, in 60% of the inoculated GCMS transgenic plant lines no systemic symptoms were recorded after 30 d.p.i. (Fig 5.4 A). Accumulation of CMV-Wem in inoculated CP+ transgenic and control hosts was followed using an immunological dot blot assay. All leaf tissue samples were taken from the top leaf, unless otherwise stated. Inoculated plants were monitored for systemic infection at 6 and 10 d.p.i. At 10 d.p.i. 20% of the inoculated plants showed virus accumulation in the top leaf, the figure increasing to 45% after 15 d.p.i (Fig. 5.6 A). At 20 d.p.i. four plants were chosen showing no virus accumulation after 15 d.p.i. Two plants showing moderate accumulation and a non-expressor (NE) were also chosen. In this experiment leaf samples were taken from the inoculated (I) leaf, the second leaf (or first leaf above the inoculated), the sixth leaf and the apical or top (T) leaf. It is evident that virus accumulated in most of the inoculated leaves of the CP+ plants but not in the systemic leaves of the top

four E plants (Fig. 5.6 B). Transgenic plant line GCMS-4 showed no virus accumulation in the inoculated leaf. The pattern of virus accumulation in the plants showing moderate protection, GCMS-2 and GCMS-23, is important as it may identify crucial steps in the infection of plants by CMV. These results seem to indicate that the virus protection mechanism, in these cases, is at the level of virus movement and not at the the initial stage of infection. However, the results from transgenic plant line GCMS-4 suggest the possibility of more than one mechanism preventing systemic infection.

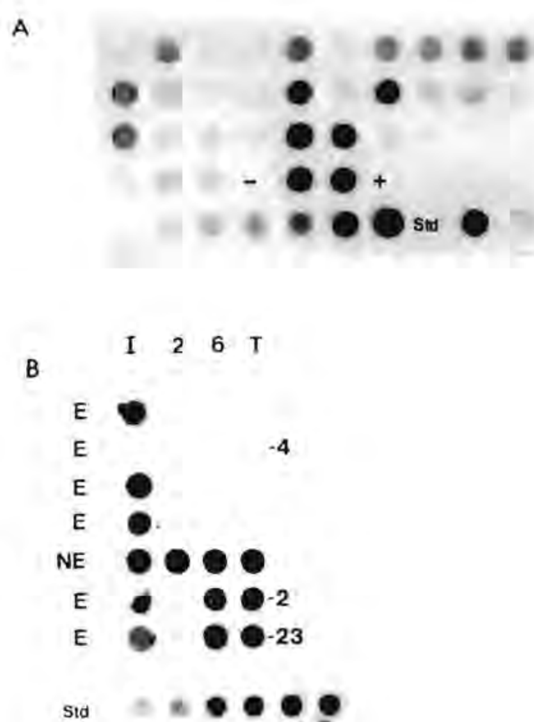

Fig. 5.6	Immuno-dot-blot detection of virus accumulation in plants expressing (E) or not expressing (NE) the CMV-Wem CP gene after inoculation of the lowest leaves with 100 µl of 1:15 dilution of CMV-Wem infected plant sap. [A] Eppendorf lid size samples were taken from the top leaves of the inoculated plants 15 days post inoculation (d.p.i.). The three negative (-) controls are samples of uninfected CP+ transgenic plant tissue. The two positive (+) controls are samples of CMV-Wem infected tobacco plant tissue. Standard consists of a 1:2 dilution of 500 ng CMV-Wem purified virus down to 15 ng. [B] Leaf samples were analysed from inoculated (I), 2nd, 6th and top (T) leaves from six E plants and one NE plant at 20 d.p.i. 10 µg of protein was loaded per dot.

TNV CP sense and antisense protection against TNV-F5P. Experiments were initiated to determine whether TNV CP sense and antisense transgenic plants could suppress symptoms caused by TNV infection, as described for a number of other viruses (see Chapter 1, Table 1.1). Preliminary testing of single leaves from individual transgenic lines, showed a number of plants with reduced necrotic lesions when compared to the controls (results not shown). Between 5 and 10 leaves from each of the potentially protected plants were subsequently assayed in the same way. Transgenic plant leaves with more lesions than half of the control number were scored as unprotected. The TNV CP+ plant lines BITS21, BITS5, GAT8 and the antisense CP lines BITA2 and BITA3 showed high protection (Fig. 5.7 and Table 5.2). There seemed to be a range of protection from low, moderate to high. A number of plants were scored as unprotected, such as line BITS14 and GAT26, while lines GAT1 and GAT7 showed moderate protection.

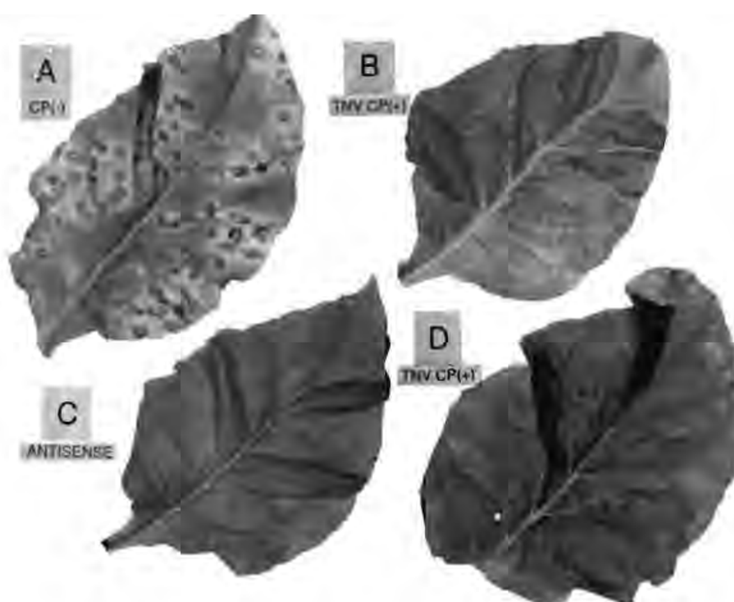

Fig. 5.7 Photograph showing the suppression of necrotic lesion numbers on individual leaves of transgenic *Nicotiana tabacum* cv Petit Havana SR1 inoculated with TNV. Leaf samples are from **A**, TNV-infected SR1; **B, C** and **D**, TNV-infected leaf from transgenic plant lines: BITS21 (B), BITA2 (C) and GAT8 (D).

Table 5.2 Effect of TNV CP sense and antisense expression by transgenic tobacco plants challenged with TNV-F5P.

Transgenic line	*Expression status*	*Lesion no.*[1]
SR1	Non-Expressor	58/10 (54)
BITS21	CP+[2]	3/ 8 (3.0)
BITS5	CP+	5/ 7 (4.7)
GAT8	CP+	8/10 (9.7)
BITA2	CP-anti[3]	17/ 5 (8.0)
BITA3	CP-anti	14/ 7 (11.1)
BITS14	CP+	60/ 5 (96.6)
GAT26	CP+	56/ 6 (32.5)
GAT1	CP+	25/ 8 (24.3)
GAT7	CP+	35/ 8 (33.3)

1 Mean number of necrotic lesions per 5-10 leaves from transgenic *Nicotiana tabacum* plants
2 CP+: transgenic plant expressing coat protein.
3 CP-anti: transgenic plant expressing the antisense coat protein transcript.
4 Sample variances.

Truncated TNV CP protection against TNV-F5P. Expression of a partial CP might aid in identifying the protection mechanism(s) involved in CP-mediated protection. The "inhibited-uncoating" hypothesis is one such proposed mechanism (see Chapter 1). The basic N-terminal arm seems to be the end of the CP required to interact with the viral RNA genome (Erickson and Rossman, 1982; Zuidema *et al.*, 1984). To investigate whether only a portion of the CP can be expressed and mediate protection against the homologous virus, 83 amino acids was deleted from the C-terminal region (see Chapter 3).

Five transgenic plants expressing the truncated TNV CP were challenged with TNV-F5P. Leaves (5-10) from each of the plants were mechanically inoculated with 100 µl of the appropriate dilution of purified TNV resulting in 50-100 lesions on control leaves. All transgenic plant lines showed a consistently reduced number of local necrotic lesions (Table 5.3), as compared to the control non-expressor (SR1). The transgenic plants tested did not however have the same high degree of protection as see in line BITS21, expressing the entire CP. These results however seem to indicate that the truncated form of the TNV CP is mediating a degree of protection.

Table 5.3 Effect of truncated TNV CP gene expression by transgenic tobacco plants challenged with TNV-F5P.

Transgenic line	*Expression status*	*Lesion no.*[1]
SR1	Non-Expressor	48/10 (62.3)[2]
BUX2	CP+(Δ)[3]	18/ 5 (6)
BUX4	CP+(Δ)	14/ 8 (28.5)
BUX8	CP+(Δ)	16/ 7 (11.4)
BUX9	CP+(Δ)	15/10 (14.6)
BUX10	CP+(Δ)	9/ 8 (42)

1 Mean number of necrotic lesions per 5-10 leaves from transgenic *Nicotiana tabacum* plants.
2 Sample variances.
3 CP+(Δ): transgenic plant expressing the truncated TNV P.

Susceptibility of double CP transgenics to mixed infection by CMV-Wem and TNV-F5P. The double transgenic plant lines CMTN were inoculated simultaneously with CMV and TNV. CMV-Wem accumulation was monitored by immunological dot blot assays at 10, 15 and 20 d.p.i. After 15 d.p.i. 60% of the transgenic plants expressing both CP genes were found to contain similar concentrations of CMV in the top leaves compared to the non- expresser SR1 control plants (Fig. 5.8 A). After 20 d.p.i. seven plants were assayed for virus accumulation in the inoculated leaf, the first leaf above the inoculated , the sixth leaf and the top leaf (Fig 5.8 B). Transgenic plant line CMTN 1.1 showed virus accumulation in the inoculated leaf and top leaf but no accumulation in the second or sixth leaves. In plant lines CMTN 4.5 and 2.5 very low CMV virus accumulation was observed even in the inoculated leaf samples.

Starting five d.p.i., plants were also scored for the development of local necrotic lesions on the TNV-F5P inoculated leaves of the CMTN doubly transgenic plants. As with the transgenic plants expressing the truncated form of the TNV CP, all TNV inoculated double transgenics showed a decrease in the number of lesions when compared with the non-expressor SR1 control plants (Table 5.4). It is interesting to note that plant line CMTN 2.5, which showed the best protection against CMV-Wem infection, also showed complete suppression in the development of necrotic lesions.

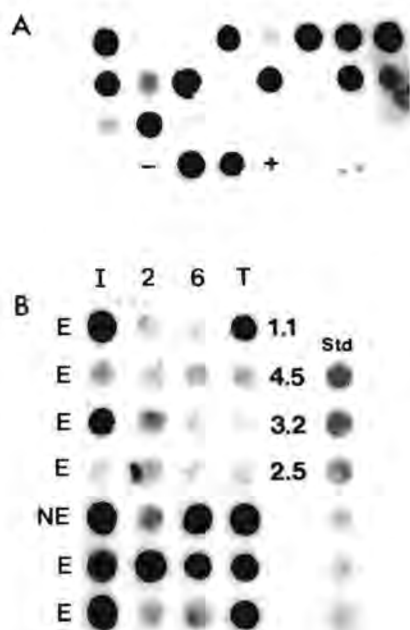

Fig. 5.8 Immuno-dot-blot detection of virus acccumulation in double transgenic plants expressing (E) or not expressing (NE) the CMV-Wem and TNV-F5P CP genes after inoculating the lowest leaves with 100 µl of 1:15 dilution of CMV-Wem infected plant sap. [A] Eppendorf lid size samples were taken from the top leaves of the inoculated plants 15 days post inoculation (d.p.i.). The two positive (+) controls and single negative (-) control are infected tobacco plant tissue and uninfected CMV CP+ transgenic tobacco plant tissue respectively. [B] Leaf samples were analysed from inoculated (I), 2nd, 6th and top (T) systemic leaves from six E plants and one NE plant at 20 d.p.i. 10 µg of protein was loaded per dot. Standard as in Fig. 5.6.

Table 5.4 Effect of the expression of the TNV CP gene in double transgenics inoculated with TNV-F5P.

Transgenic lines	*Expression status*	*Lesion no[1].*
SR1	Non-Expressor	33 (8.6)[3]
CMTN 1.1	CP+[2]	8 (6.3)
CMTN 4.5	CP+	2 (1.6)
CMTN 3.2	CP+	10 (10.2)
CMTN 2.5	CP+	0 (0.3)
CMTN 7.2	CP+	16 (7.0)
CMTN 7.3	CP+	11 (2.0)

1 Mean number of necrotic lesions per 3 leaves
2 Doubly transformed *Nicotiana tabacum* expressing both the CMV and TNV CP genes.
3 Sample variances.

5.4 DISCUSSION

In this chapter the transformation of tobacco leaf discs of *Nicotiana tabacum* cv Petit Havana line SR1 with the CP genes of CMV and TNV has been described, as well as the subsequent analysis of the degree of resistance of transformants to infection by CMV and TNV. Both CMV and TNV are economically important plant viruses because of their world-wide distribution, extensive host ranges and their effects on yields of agriculturally important crop plants. Studies in South Africa have shown that a complex of viruses including CMV and TNV are present in a number of natural infections (von Wechmar *et al.*, 1990). Due to the already high and increasing prevalence of both viruses, transgenic plants were engineered to express both the CMV and TNV CP genes in a single plant, in order to protect against this mixed virus infection. Many reports describe "coat protein-mediated resistance" (Table 1.1, Chapter 1). Since the demonstration that resistance in tobacco to TMV infection is directly mediated by the CP and not by its mRNA (Powell *et al.*, 1990), it has usually been accepted that the same is true in most cases where genetically engineered resistance has been achieved using a CP gene. However for an increasing number of cases protein-mediated protection is not evident (Kawchuk *et al.*, 1990, 1991; van der Vlugt *et al.*, 1992; Lindbo and Dougherty, 1992; Fang and Grumet, 1993) and other mechanisms have been proposed. It seems that no general rule can be made concerning the possible effectiveness of a certain CP gene in CP-mediated resistance, which therefore indicates that it is necessary to fully investigate each transgenic plant line established expressing the CP gene or a form thereof.

CMV resistance

Published work indicates that transgenic tobacco plants expressing the CP gene of CMV-D, -WL, -C, -O and -Y strains are protected against CMV infection (Cuozzo *et al.*, 1988; Namba *et al.*, 1991; Quemada *et al.*, 1991; Nakajima *et al.*, 1993; Okuno *et al.*, 1993). Recently Yie *et al.* (1992) reported a high level of resistance to CMV conferred by CMV CP and satellite RNA expression in single transgenic plants. However, transgenic plants expressing CMV CP antisense transcripts have not been as well investigated: in the only report to date, Cuozzo *et al.* (1988) showed that transgenic plants expressing the CMV CP antisense transcript were protected from CMV infection, but only at low inoculum concentrations. In order to reassess the potential of antisense CP mRNA as a means of plant virus control, the entire CMV-Wem CP gene including a large portion of both the 5'- and 3'-untranslated regions was expressed in the antisense

orientation. Without performing complete dose or response curves, the results from my work show significant protection from CMV infection induced by CMV-Wem CP and CP antisense transcript expression. Several CP+ and CP-antisense tobacco lines were challenged with CMV-Wem. More than 50% of the CP+ and CP-antisense plants did not develop disease symptoms 30 d.p.i. In either case, the CMV-Wem inoculum concentration was not increased in order to determine the virus concentration above which the resistance is overcome; however resistance was obtained with both sense and antisense transgenics at the lowest dilution of CMV infected plant sap which resulted in systemic symptoms on all control plants only a few days post inoculation. From the low level of protection in the antisense plants and its dependence on inoculum concentration, Cuozzo *et al.* (1988) suggested that antisense-CP RNA is not as effective as the CP in prevention of viral infection. The preliminary results obtained here however suggest that, in this case, antisense CP RNA mediated protection is comparable to that seen for plants expressing the CP. After inoculation of CP+ plants with CMV-Wem it was evident from the immuno-dot-blot results that the accumulation of virus in the inoculated and systemically-infected leaves was delayed compared to the controls. The results obtained for the same CMV infected CP+ (E) plants 20 d.p.i. showed three altered symptom phenotypes when compared to the non-expressor (NE) (see Fig. 5.6B). The level of virus accumulation in the inoculated leaf of most of the CP+ transgenics was observed to be the same as in the control plants, and not significantly reduced as previously reported for CMV (Cuozzo *et al.*, 1988) and TMV (Nelson *et al.*, 1987). However, a single CP+ transgenic line (GCMS-4) was highly protected from infection with CMV, showing no accumulation of virus in either the inoculated leaf or the systemically-infected leaves. Interestingly, plant lines GCMS-2 and GCMS-23 (Fig 5.6B), which showed less of a delay in the development of systemic disease symptoms 20 d.p.i., accumulated virus in the top and 6th leaves as well as the inoculated leaves, but did not show accumulation in the 2nd leaves. These results indicate that virus movement through the plant occurred from the inoculated leaf to the apical shoot tip, and then back to the lower fully expanded leaves. Non-transformed SR1 plants showed a similar virus infection pattern (results not shown). This may be understood in the light of the reports of Leisner *et al.* (1993), who showed that once viruses invade the vascular system they are swept along with the flow of photoassimilates from source leaves to sink leaves. This means that in systemic infections young leaves import viruses from inoculated leaves whereas mature leaves do not (Leisner *et al.*, 1993).

Previous reports have also shown that transgenic tobacco plants expressing the CP of CMV are protected to a significant level from infection by various CMV strains (Namba *et al.*, 1991; Quemada *et al.*, 1991; Nakajima *et al.*, 1993). For this reason and the fact that details of protein structure affecting serological relatedness can play a role in cross-protection (Wen *et al.*, 1991) the most resistant CMV-Wem CP+ R_0 tobacco lines were challenged with CMV-Q and CMV-Y. The CMV CP+ lines showed excellent protection against CMV-Q, which belongs to the homologous subgroup. In contrast, only a delay in systemic symptom development could be observed with CMV-Y infection (heterologous subgroup). However, in these plants only leaves higher up the plant from the inoculated leaf became systemically infected: these higher leaves displayed symptoms of varying degrees of severity. In control plants the inoculated leaves showed severe symptom development. It has been found that plants expressing the CMV-WL CP gene showed a broad spectrum of protection against various CMV strains (Namba *et al.*, 1991). One might expect that as the CP sequence of CMV-Wem has a 98% amino acid sequence identity with that of CMV-WL, plants transgenic for this protein could be expected to be similarly protected. However, the level of CP-mediated resistance against the heterologous CMV-Y strain is not nearly as high as 85-90% found for CMV-Q. (see Fig. 5.4A & B).

TNV resistance

As described above the CMV-Wem CP+ and CP-antisense transgenic plants showed two manifestations of resistance, that of a delay or absence of systemic disease development and a reduction in virus accumulation in the infected CP+ plants compared to infected non-transgenic controls. A third manifestation of CP-mediated resistance is the reduction in the number of sites where infection occurs. TNV-F5P infections in mechanically inoculated transgenic plants expressing either the full-length TNV CP, the CP antisense transcript or a truncated form of the CP (CP), showed a significant reduction in symptom severity (see Tables 5.2 & 5.3). The altered symptom phenotypes ranged from near wild type susceptibility to apparent immunity.

The transgenic plants expressing TNV CP did not not show a higher degree of resistance than plants expressing the full-length CP. This is in contrast to the report that truncated forms of the TEV CP are more effective in CP-mediated resistance to TEV than transgenic plants that express the full-length TEV CP (Lindbo and Dougherty, 1992); however, TNV and TEV are very different viruses, with different morphologies, assembly mechanisms and biology, so it is

not entirely unexpected that the same rules should not apply in the two cases. The TNV CP has a basic N-terminal arm (Meulewaeter *et al.*, 1990; Coutts *et al.*, 1991), similar to AlMV, whose "arm" has been shown to interact with the viral RNA genome (Zuidema *et al.*, 1984). This led to the speculation that in order for the CP to mediate resistance via the proposed "inhibited-uncoating" hypothesis, this viral RNA binding domain would be required. However from this study it remains unclear whether just the truncated form of the CP or specifically the N-terminus is required for the virus resistance observed, since no control plants were challenged which expressed only the C-terminus of the TNV CP. Lindbo and Dougherty (1992) reported that with TEV, C-terminal and N- and C-terminal truncated products were more effective in CP-mediated resistance than the whole protein. In contrast to these results Fang and Grumet (1993) observed ineffective protection against zucchini yellow mosaic Potyvirus (ZYMV) in transgenic plants expressing the highly conserved core region (conserved central- and carboxy-terminal region) of the ZYMV CP. The reason(s) for the difference between these experiments with morphologically-similar Potyviruses is unclear. Lindbo and Dougherty (1992) speculate that the truncated CPs are in some way dysfunctional and are more effective at disrupting the normal virus-host relationship than the full-length CP. From this preliminary study, it appears that transgenic plants expressing defective proteins could be among the most effective virus control strategies. It is also apparent, from this and other published work, that there is no firm rule governing whether or not truncated CPs may be effective in conferring resistance, even in viruses of the same taxanomic group.

Resistance conferred in double transgenics

Lawson *et al.*, 1990 were the first group to demonstrate that it is possible to engineer resistance to two different viruses. They evaluated several different transgenic lines expressing PVX and PVY CP and identified a single transgenic that was essentially immune to infection by PVX and PVY. Doubly transgenic plants expressing both the CMV and TNV CPs were coinoculated with CMV-Wem and TNV-F5P. It was found that two double transgenic lines, CMTN 2.5 and 4.5, displayed significant resistance to CMV-Wem infection. The levels of CMV accumulation was reduced in both the inoculated leaf and leaves above the inoculated leaf in the two plant lines. (Fig 5.8B). Plant line CMTN 2.5 also showed complete suppression in the development of necrotic lesions as a result of infection by TNV (Table 5.4). The level of protection achieved in this plant

against both viruses is promising and may represent an effective way of generating resistance to this mixed virus infection.

CP-mediated resistance mechanisms

The delay or complete absence of virus accumulation in inoculated leaves of the CP+ plants or its complete absence (GMCS-4: Fig. 5.6B) suggests that infection is prevented in these leaves. As previously suggested for TMV and AlMV, this could result from prevention of virus uncoating or uptake (Nelson *et al.*, 1987; Tumer *et al.*, 1987). Earlier cross-protection studies with CMV (Dodds *et al.*, 1985) showed that protection broke down when the challenge inoculum was CMV RNA. These results suggested that the protection resulted from inhibition of uncoating, although this did not rule out other possibilities such as inhibition of virus uptake. Absence of resistance to infection by TMV RNA or by partially decapsidated virions (Register and Beachy, 1988) and other experiments (Osbourn *et al.*, 1989a and 1989b; Mundry *et al.*, 1991) has also pointed to the primary effect of protection in TMV CP expressing plants being at a very early event in the infection process, presumably decapsidation. I propose that in the case of the CMV CP+ transgenics, the observed CP-mediated resistance is due not only to interference with, or inhibition of, an early event in the infection process, but possibly occurs in combination with a later event; that of a delay in virus movement from cell to cell and also in long distance movement. However, I cannot discount the fact that CP-mediated resistance could result from several sites of CP or CP mRNA interference, or that several different but additive mechanisms are present. What is evident from the results, in most cases, is comparable to a natural viral-host plant interaction known as "subliminal infection", where individual cells are able to support viral replication but the virus is unable to move to adjacent cells. In this case the host appears to be resistant to the virus (Hull, 1989; Deom *et al.*, 1992).

In most of the transgenic CP+ plants, challenge virus replication is seen not to be totally inhibited, but there is a delay in cellular virus accumulation in the inoculated leaves which results in an overall delay in the development of systemic symptoms. This might serve to explain the reduction or absense of virus in the systemically-infected leaves of CMV CP+ plants (Fig. 5.4A). The expressed CP may be inducing a type of subliminal infection. Following entry and replication, progeny viruses may not be capable of moving into adjacent healthy cells, or are delayed in there movement due to a threshold barrier needing to be overcome (Chapman *et al.*, 1992; J. Atabekov personal

communication). From the results (Figs. 5.6B & 5.8B) there is evidence that the challenge virus is slow in replicating to sufficient levels in the inoculated leaves in order to reach the threshold level necessary for cell-to-cell or long distance movement.

The CPs of many viruses may control viral RNA synthesis or stability (Chapman *et al.*, 1992). For AlMV the coat protein is a *trans*-acting factor that both increases positive-strand RNA accumulation and decreases negative strand RNA accumulation (van der Kuyl *et al.*, 1991). Although there is no direct evidence that the same is true for CMV, I propose that endogenously expressed CP may interfere with viral RNA regulation thus disrupting replication and virus accumulation. This could be an added factor apart from possible inhibition of uncoating, culminating in the CP-mediated resistance demonstrated. The availability of CP-producing plants now makes it possible to test this function.

No formal experimental correlation was made between the level of CP or CP mRNA transcripts expressed, and the level of resistance among the different transgenic lines. Although from the western blot results plant line GCMS-8 was found to accumulate the highest CP levels, this plant line did not show the highest degree of resistance when challenged with CMV-Wem. Similar results have been reported for CMV-WL (Namba *et al.*, 1991), suggesting that CP concentration in transgenic plants does not appear to be a useful predictor of the degree of protection. With this in mind it is perhaps important to note the expression pattern of the CaMV 35S promoter: this has been found to consist of several elements that control developmentally regulated and tissue-specific expression in plants (Jefferson *et al.*, 1987; Benfey *et al.*, 1990a and 1990b). Transcriptional expression of integrated CP genes may therefore differ both in time and in particular tissues among the different transgenic lines during their development. This may explain the variable levels of resistance found. This implies that irrespective of the level of CP gene expression, each transgenic plant line should be tested for the presence and level of resistance.

From these studies it has remained unclear whether the CP encoding RNA or the protein itself is responsible for the the virus resistance observed in both the CMV and TNV resistant transgenic plants, since no control plants were produced which only produced viral CP sense transcripts. What is evident however is that these results reflect more than one mechanism involved in the protection.

CP-antisense mechanisms

CP RNAs are not thought to be ideal targets for antisense technology since they tend to be expressed at higher levels than other viral genes and are not always essential for virus replication and symptom development (Bejarano and Lichtenstein, 1992) A number of studies with transgenic plants expressing plant viral antisense RNAs have shown only minimal protection, and then at only low concentrations of challenge inoculum (Hemenway *et al.*, 1988; Cuozzo *et al.*, 1988; Rezaian *et al.*, 1988). However, transgenic plants expressing antisense RNA to PLRV CP gave similar resistance to those expressing the sense CP RNA (Kawchuk *et al.*, 1991; van der Wilk *et al.*, 1991). The results presented here for CMV (see Fig. 5.4A) and TNV (see Table 5.2) antisense CP mediated resistance are also promising; however further investigations are required to determine the full effectiveness of this strategy in both virus systems.

Several mechanisms can be proposed to explain the differences in disease development between the controls and the CP-antisense plants. One possibility is the inhibition of CP synthesis resulting through the formation of an antisense-sense RNA duplex. There are however a number of arguments against this proposal. The large volume of the cytoplasm may result in an increase in time required for duplex interactions, and the antisense RNA may be degraded by RNases before encountering its target (Bejarano and Lichtenstein, 1992). Moreover, in the case of CMV and TNV there is evidence that membrane bound virus-specific vesicles associated with the tonoplast may be the sites of viral RNA synthesis (Hatta and Francki, 1981). For this reason one could speculate that the replicating viral RNA would be relatively inaccessible as a target for the antisense RNA. An alternative possibility is that the antisense message can compete with viral proteins or host factors essential for replication of the virus, since negative strand RNA is normally produced during viral replication and has to interact with the necessary factors to serve as a template for positive strand synthesis. In the case of CMV CP-antisense plants, the entire 5'- and a large portion of the 3'-untranslated region were included in the antisense construct to be expressed. As these regions are known to be involved in the initiation of transcription of plus-sense templates and RNA accumulation (Davies and Symons, 1988), it should be reasonable to assume that by including both regions may have an increased effect on reducing challenge virus replication.

In summary, I have demonstrated that transgenic tobacco plants expressing forms of CMV and TNV CP, independently or together, were protected against

CMV and TNV infection. The results presented here extend the demonstration of genetically engineered CP- mediated protection to another plant virus class, the Necroviruses, of which TNV is a member. In the CMV-Wem CP+ and CP-antisense transgenic plants there was a significant delay in systemic CMV disease development with apparent immunity observed in more than half of the tested plants. In the CMV CP+ plants, virus accumulation was delayed and some plants showed little or no CMV accumulation. Plants expressing either the TNV CP, CP-antisense transcript or a truncated form of the CP also showed a reduction in symptom severity.

This is the second report of the expression of two viral CP genes in a single transgenic plant, thereby engineering resistance to a mixed virus infection, or either virus individually. Similar levels of protection to those described above were displayed in these doubly transgenic plants. Although virus multiplication was not completely hindered in every transgenic plant, in some cases it declined to very low or undetectable levels. Thus by using these methods the quality and yield of crop plants in the environment in which mixed infections occur should be improved. Another possible outcome of the use of such transgenics could be the reduction or effective elimination of CMV through aphid transmission, in natural infections.

It is hoped that the significant results obtained in the case of the CP-antisense plants do not change dramatically under further investigation, as there may be an advantage in using the CP-antisense transcript instead of the coat protein to achieve viral resistance. Heterologous encapsidations are observed during natural mixed infection (Waterhouse *et al.*, 1988; Bourdin and Lecoq, 1991; Wen *et al.*, 1991) and transcapsidation of a virus by transgenic CP has been demonstrated for TMV (Osbourn *et al.*, 1990) and PVY (Farinelli *et al.*, 1992). This may prove undesirable in transgenic plants (de Zoeten, 1991). The reason for is that transcapsidation has led to altered virus-vector specificities in nature (Rochow, 1970; Creamer and Falk, 1990). de Zoeten (1991) suggests that since transcapsidation or phenotypic mixing may change the vector range of a virus, this encapsidation could lead to the creation of an apparently "new" disease. If a different aphid or vector has a plant-host range distinct from those of the usual vector, transcapsidated or phenotypically mixed capsidated nucleic acid could thus be introduced into a "new" host range, where it can initiate replication. Thus this nucleic acid could become transmissible by a vector species to a plant-host range, both quite different from the plant and vector involved in the original

infection. Such a phenomenon would not occur in plants expressing CP-antisense transcripts. The same would be true for plants expressing truncated or dysfunctional CPs and would presumably give them a commercial advantage as a result of their possible minimum ecological impact.

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ABBREVIATIONS

General

A	adenine / adenosine
Amp	ampicillin
ATP	adenosine 5'-triphosphate
ATG	methione / start codon
BA	benylamino purine
β-gal	beta-galactosidase
BCIP	5-bromo-4-chloro-3-indolyl phosphate
bp	base pair
BSA	bovine serum albumin
C	cytidine / cytosine
Cb^R	carbenicillin resistance
cDNA	complementary DNA
c.f.u.	colony forming units
°C	degrees centigrade
CIP	calf intestinal alkaline phosphatase
CP	coat protein
CP+	transgenic plants expressing the CP
cv.	cultivar
Da	Dalton
dCTP	deoxycytidine triphosphate
dATP	deoxyadenosine triphosphate
dUTP	deoxyuracil triphosphate
d.p.i.	days post inoculation
DIG	digoxigenin
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
ds	double stranded
E	CP expressor
EDTA	ethylenediaminetetraacetic acid (disodium salt)
EtOH	ethanol
Fp	forward primer
g	gram
g	gravitational acceleration
G	guanine / guanosine
GCG	Genetics Computer Group
h	hour
Hyg	hygromycin
IgG	gamma-immunoglobulin
IPTG	isopropylthio-b-D-galactoside
Km	kanamycin
kb	kilobase
KPa	kilopascals
LB	Luria broth
M	Molar
mal	maltose
MBP	maltose binding protein
MBP::CMV CP	maltose binding protein and CMV coat protein fusion
MBP::TNV CP	maltose binding protein and TNV coat protein fusion
min	minute
MIC	minimum inhibitory concentration
ml	milliliter

Mol	Mole
MS	Murashige and Skoog salts
mRNA	messenger RNA
M_r	relative molecular weight
NAA	naphthalene acetic acid
NBT	nitro-blue tetrazolium chloride
NE	non expressor of CP
nm	nanometer
nt	nucleotide
OD_{600}	optical density at 600 nm wavelength
O/N	overnight
ORF	open reading frame
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
R_o	first plant transformant (regenerant)
rpm	revolutions per minute
RNA	ribonucleic acid
Rp	reverse primer
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
sec	second
ss	single strandeed
Tl	T-DNA left border integration sequence
Tr	T-DNA right border integration sequence
T	thymine / thymidine
TAG	amber stop codon
T-DNA	Ti plasmid DNA
TE	tris-EDTA
Ti	tumour inducing
Tris	tris(hydroxymethyl)aminomethane
t-RNA	transfer RNA
TGA	stop codon
U	unit
uv	ultraviolet
vir	virulence
v/v	volume per volume (in ml per 100 ml)
w/v	weight per volume (in g per 100 ml)
Wem	CMV Wemmershoek isolate
wk	week
X-gal	5-bromo-4-chloro-3-indolyl-b-D-galactoside
α-	alpha
β-	beta
γ-	gamma
λ-	lambda
μ	micro

List of virus acronyms

AlMV	Alfalfa mosaic virus
ArMV	Arabis mosaic virus
BMV	Brome mosaic virus
BNYVV	Beet necrotic yellow vein virus
CaMV	Cauliflower mosaic virus
CarMV	Carnation mottle virus
CMV	Cucumber mosaic virus
CMMV	Chrysanthemum mild mottle virus
CNV	Cucumber necrosis virus
CyRSV	Cymbidium ringspot virus

GCMV	Grapevine chrome mosaic virus
ORSV	Ondontoglossum ringspot virus
PEBV	Pea early browing virus
PeMV	Pepper mottle virus
PLRV	potato leaf roll virus
PMMV	Pepper mild mottle virus
PPV	Plum pox virus
PRV	Papaya ringspot virus
PVM	Potato virus M
PVS	Potato virus S
PVX	Potato virus X
PVY	Potato virus Y
RMV	Ribgrass mosaic virus
RRV	Raspberry ringspot virus
RSV	Rice stripe virus
SBMV	Southern bean mosaic virus
SHMV	Sunn hemp mosaic virus
SMV	Soybean mosaic virus
TCV	Turnip crinkle virus
TEV	Tobacco etch virus
TMGMV	Tobacco mild green mosaic virus
TMV	Tobacco mosaic virus
TNV	Tobacco necrosis virus
ToMV	Tomato mosaic virus
TRV	Tobacco rattle virus
TSV	Tobacco streak virus
TSWV	Tomato spotted wilt virus
ZYMV	Zucchini yellow mosaic virus