ANALYSIS AND APPLICATION OF CaMV 35S PROMOTER-DRIVEN CLONES OF TOBACCO MOSAIC VIRUS

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by

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Dedicated to my Parents and Ann

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Please celebrate the completion of this thesis with me.

ABSTRACT

Recently, cDNA clones of RNA viruses have been fused to the 35S promoter from CaMV. Having received a 35S-cDNA clone of TMV-U1 we investigated its infectivity. Protoplast, microprojectile bombardment and plant transformation experiments were conducted. We were able to demonstrate that the clone was highly infectious to tissue known to host TMV. However, the 35S-TMV cDNA construct was unable to reliably induce an infection following the manual inoculation of leaves, possible reasons have been discussed. Using the 35S-TMV cDNA we generated four replication-marker constructs. Each construct contained the luciferase marker gene inserted in place of the TMV coat protein ORF. As a result, the constructs should have been capable of replication-dependent expression of luciferase. The constructs were tested using microprojectile bombardment and plant transformation experiments. Unfortunately the constructs did not appear to function in the manner intended. The design of each construct has been fully discussed and we conclude that further analysis of transgenic plants lines is required. The application of TMV-based replication and replicase constructs for studies of *Tm-1* and *N* gene mediated resistance has been considered. Resistance to the *N* gene has been investigated via microprojectile bombardment and plant transformation experiments. Evidence suggests that the formation of an active replicase-complex may be required to elicit the *N* gene-mediated hypersensitive response. The expression of TMV replicase proteins alone does not appear to elicit the response. Following the inoculation of TMV on to transgenic *N. tabacum* Samsun *NN* plants, expressing replication or replicase constructs, unusual phenotypes were observed. A number of interesting transgenic plant lines have been generated. Investigations have been conducted to determine whether they were resistant to TMV. Results suggest that *N. tabacum* SR1 plants expressing a TMV-based replicase construct exhibit a resistant phenotype.

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ABBREVIATIONS AND ACRONYMS

Many abbreviations and acronyms used within this thesis are explained the first time that they appear in the text. In addition the majority are defined within the following lists. These lists are not exhaustive and in particular lack abbreviations for well known units of measurement. Abbreviationx excluded from the lists are defined and recommended for use by the journal Plant Physiology.

VIRUSES

AlMV	alfalfa mosaic virus
BMV	brome mosaic *Bromovirus*
BNYVV	beet necrotic yellow vein *Furovirus*
BSMV	barley stripe mosaic *Hordeivirus*
CaMV	cauliflower mosaic *Caulimovirus*
CERV	carnation etched ring *Caulimovirus*
CGMMV	cucumber green mottle mosaic *Tobamovirus*
CMV	cucumber mosaic *Cucumovirus*
CPMV	cowpea mosaic *Comovirus*
CSSV	cacao swollen shoot *Badnavirus*
GTAMV	green tomato atypical mosaic *Tobamovirus*
ORSV	odontoglossom ringspot *Tobamovirus*
PEBV	pea early-browning *Tobravirus*
PHV	pepper huassteco virus *Geminivirus*
PPV	plum pox *Potyvirus*
PVX	potato X *Potexvirus*
RGM	Ribgrass mosaic *Tobamovirus*
TEV	tobacco etch *Potyvirus*
TMGMV	tobacco mild green mosaic *Tobamovirus*
TMV	tobacco mosaic *Tobamovirus*
TNV	tobacco necrosis *Necrovirus*
TRV	tobacco rattle *Tobravirus*
TYMV	tobacco yellow mosaic *Tymovirus*
ZYMV	zucchini yellow mosaic *Potyvirus*

PSTV	potato spindle tuber viroid
TMV-strain	particular strain or isolate (eg. TMV-U1; TMV-L)

BIOLOGICAL

Ω	TMV 5' untranslated region
A	adenine
BS	bluescript
C	cytosine
CAT	chloroamphenicol acetyl transferase
cDNA	complementary DNA
CP	coat protein
cv.	cultivar
DHFR	dihydrofolate reductase
DNA	deoxyribonucleic acid
ds	double-stranded
G	guanine
GFP	green fluorescent protein
GUS	β-glucuronidase (gene/protein)
HR	hypersensitive responce
I_n-RNA	Intermediate length RNA (n= 1 or 2)
lac Z	β-Galactosidase
LMC-RNA	low molecular weight component RNA
LUC	luciferase (gene/protein)
M^7GpppG	7-methyl guanosine
mRNA	messenger RNA
NPT	neomycin phosphotransferase
nsP	non-structural protein
OAS	origin of assembly
ORF	open reading frame
p	plasmid
PR	pathogen related
RAPD	random amplified polymorphic DNA
rDNA	ribosomal DNA
RF	replicative form
RFLP	restriction fragment length polymorphism
RI	replicative intermediate
RNA	ribonucleic acid
35S	promoter for CaMV 35S RNA
SGP	subgenomic promoter
ss	single-stranded
T	thymine

T_0	transgenic plant, original transformant
T_1	transgenic plant, first generation
T-DNA	transfered DNA
Ti	tumor inducing
tRNA	transfer RNA
U	uracil
UTR	untranslated region
vir	virulence gene
vRNP	virus-specific ribonucleoprotein particle

CHEMICALS/TECHNIQUES

ATP	adenosine triphosphate
6-BAP	6-Benzylaminopurine
BCIP	5-bromo-4-chloro-indolyl phosphate
BSA	bovine serum albumin
DEPC	diethyl pyrocarbonate
dH_20	distilled water
dNTP	deoxyribonucleotide triphosphate
DTT	Dithiothreitol
EDTA	ethylenediaminetetraacetic acid
HEPES	N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulphonic acid)
IAA	indole-3-acetic acid
IPTG	isopropylthio-β-D-galactoside
LA/B	Luria-Bertani agar/broth
MES	2-(N-morpholino)ethanesulphonic acid
MS	Murashige and Skoog
4MU	4-methyl umbelliferone
MUG	methylumbelliferyl β-Glucuronide
NAA	naphthaleneacetic acid
NA/B	nutrient agar/broth
NBT	nitroblue tetrazolium
OLB	oligo labelling buffer
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PEG	polyethylene glycol
PMSF	Phenylmethylsulphonyl fluoride
PVP	polyvinylpyrrolidone

rNTP	ribonucleotide triphosphate
sarcosyl	N-lauroylsarcosine
SDS	sodium dodecyl sulphate
SSC	sodium chloride sodium citrate
TAE	tris-acetate EDTA
TBE	tris-borate EDTA
TBS	Tris buffered saline
TCA	trichloroacetic acid
TEMED	N,N,N',N'-tetramethylethylenediamine
Tris	tris(hydroxymethyl)aminomethane
Tween 20	Polyoxyethylene-sorbitan monolaurate
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside
X-GLUC	5-bromo-4-chloro-3-indoyl-β-glucorinide

MISCELLANEOUS

~	approximately
A	absorbance
bp	base pair
Da	dalton
dpb	days post bombardment
FU	fluorescent units
kb	kilobase
kDa	kilodalton
LU	light units
M_r	relative molecular mass
S	sedimentation coefficient
t	time
v	volts
v/v	volume:volume
w/v	weight:volume
x*g*	times gravity

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LIST OF TRANSGENIC PLANT LINES

U	*N. tabacum* SR1 transformed with the infectious construct from pB-35STMV
L	*N. tabacum* SR1 transformed with the replicase-marker construct from pBU-S4-L1
T	*N. tabacum* SR1 transformed with the replication-marker construct from pBU-SLU
C	*N. tabacum* SR1 transformed with the replication-marker construct from pBU-SLLUTR
X	*N. tabacum* SR1 transformed with the replication construct from pBU-XM
B	*N. tabacum* SR1 transformed with the replicase construct from pBTRepTerm
M	*N. tabacum* Samsun *NN* transformed with the replication construct from pBU-XM
R	*N. tabacum* Samsun *NN* transformed with the replicase construct from pBTRepTerm
G	*N. tabacum* Samsun *NN* transformed with the 35S-GUS construct from pMKC 6

CHAPTER 1

INTRODUCTION

1.1 A BRIEF HISTORY OF TMV

Tobacco mosaic virus (TMV) is neither the most commercially important nor the most widespread plant virus. However since first described by Mayer in 1886 it has received much attention. This is in part due to the stability of virus particles and its ability to multiply to high titres within host plants. Infected cells can contain 10^5-10^6 virus particles and at 2-3 days post infection the synthesis of viral proteins can account for ~10 % of total protein synthesis (Matthews, 1991; Matthews, 1992).

Few research proposals offer the appeal of sailing around the coast of West Africa in order to classify different viruses according to their hosts and the mosaic symptoms which they induced. However, such a trip was reported by McKinney (1929). In the same year Holmes observed that, in addition to mosaic symptoms, TMV was capable of inducing necrosis on some plants. Furthermore, the necrotic lesions which developed on *Nicotiana glutinosa* were found to be useful for determining virus concentration. It was soon realised that *N. glutinosa* contained a resistance gene which could possibly be exploited for crop protection (Holmes, 1938). While investigations concerning this gene continued other resistance genes were also identified. In particular genes were identified which conferred resistance to *Lycopersicon ecsculentum* (Clayberg *et al.*, 1960). Efforts made by plant breeders to exploit resistance genes for crop protection resulted in varying degrees of success. Researchers continued to investigate the physical, biological and chemical properties of different strains of TMV (see review by van Regenmortel, 1981). As a result the anatomy of TMV particles was established and studies to investigate the assembly of RNA and coat protein into particles commenced. The development of purification techniques led to the identification of a number of RNA species associated with viral replication and gene expression (e.g. Nilsson-Tillgren *et al.*, 1970; Jackson *et al.*, 1972). As a result proteins encoded by the viral genome were identified (e.g. Patterson and Knight, 1975; Bruening *et al.*, 1976). Studies of TMV gene expression and protein function continued through the 1980's and into the 1990's. The ability to prepare complementary DNA (cDNA) copies of the RNA genome (Dawson *et al.*, 1986; Meshi *et al.*, 1986) was a significant breakthrough and allowed the application of many molecular techniques. While much has been learnt, investigations need to be continued in order to further the understanding of TMV.

It is not possible to describe all investigations involving TMV, or pay tribute to all researchers, within this introduction. However, it is hoped that the information presented will provide an introduction to the virus, current research interests and the subject of host resistance.

1.2 CLASSIFICATION

The classification of plant viruses is not an easy task and has been the subject of much debate (Siegel and Wildman, 1954; Francki, 1981). Plant viruses are currently classified into over 25 different genera according to their host range, the appearance of particles, amino acid and nucleotide sequences, antigenic properties and symptom development on diagnostic plants. The majority of genera consist of viruses with single-stranded RNA (ssRNA) genomes. TMV is the type member of the *Tobamovirus* genus (reviewed by van Regenmortel, 1981). The particles form ridged rods, 300 nm long and 18 nm wide. Genetic information is carried on a single-strand of positive-sense RNA. This genomic RNA consists of ~6400 nucleotides and has a relative molecular weight (M_r) of $2x10^6$ Da. It is arranged in a spiral and coated by ~2 000 coat protein subunits. Throughout the centre of each particle runs a hollow channel 4 nm in diameter. Although variations exist between different tobamoviruses they share a similar structure and genomic organisation (van Regenmortel, 1981; Dawson and Lehto, 1990). Some confusion exists about which of the many genus members should be classified as strains of TMV and which belong to separate sub-genera. Two common strains (also referred to as *vulgare*) have been isolated. TMV-U1 is an American isolate and has been fully sequenced (Goelet *et al.*, 1982). The original sequence data suggested that two variants existed. Polymorphism of this kind is not common to plant viruses and the theory has since been discredited (Meshi *et al.*, 1983; Dawson *et al.*, 1986). The second common strain was isolated in Japan and named TMV-OM. Restrictions maps demonstrated that TMV-OM was very similar to TMV-U1 (Meshi *et al.*, 1982b). As the name suggest TMV infects, but is not limited to, *Nicotiana* plants. The tomato strains of TMV (also referred to as ToMV) are able to infect *Nicotiana* plants but are more commonly isolated from *Lycopersicon* species (*Nicotiana* and *Lycopersicon* are both members of the Solanaceae family) The most well studied tomato strain is a Japanese isolate known as TMV-L. Sequence data shows that TMV-L is 80 % similar to TMV-U1 (Goelet *et al.*, 1982; Ohno *et al.*, 1984). The differences that do exist must account for the individual host preference of each virus. In addition to TMV-L, there are many other tomato strains. In particular, investigations have been conducted using a number of Ohio isolates (Pelham, 1972). Other tobamoviruses differ from TMV-U1 to a greater extent. For example, there are considerable differences between the common strains and the cowpea strain (TMV-Cc) (Meshi *et al.*, 1982a; Meshi *et al.*, 1982b); while cucumber green mottle mosaic *Tobamovirus* (CGMMV) is similar in some respects to the common strains, it shares similarities with TMV-Cc (Meshi *et al.*, 1983b).

1.3 THE DISEASE

It has been estimated that ~ 5 % of crops are lost due to disease and one third of these are due to plant viruses (Fraser, 1992). TMV is mechanically spread throughout ~ 200 crop species world wide (Dawson and Lehto, 1990). While symptoms can vary, infection by TMV commonly leads to unhealthy stunted and withered plants and as a result crop yield is reduced. Where infected fruits develop their colour, texture and flavour can be poor.

When studying symptoms care must be taken to ensure that they are due to virus infections and not other environmental factors. The symptoms are a result of a number of complex interactions (reviewed by Culver *et al.*, 1991 and discussed in Sections 1.7.1 and 1.7.3). The way in which they develop is dependent on the strain of TMV and the host plant infected. Typical mosaic symptoms can be observed following the infection of *N. tabacum* SR1 with TMV-U1. The mosaic pattern, of dark green islands in a light green background, develops on systemically infected leaves which are <1.5 cm long at the time of infection (Culver *et al.*, 1991). The chlorosis is thought to be due to interactions between the virus and developing chloroplasts (Sections 1.7.1; 1.7.3). Plants have been regenerated from both dark and light green tissue (Murakishi and Carlson, 1976). The plants originating from the light green areas were infected with TMV while many of the plants regenerated from the dark green areas were virus free. Furthermore, the young virus free plants demonstrated resistance to TMV. This transient resistance was gradually lost as the plants matured (Murakishi and Carlson, 1976). It was suggested that some cells in the leaf primordia were resistant to TMV, cell division therefore led to clusters of the resistant cells. The mechanism behind this type of resistance remains unknown. A different type of chlorosis is sometimes observed on inoculated fully developed leaves. It is thought to be due to interactions between the virus and mature chloroplasts (Section 1.7.3). Normally TMV-U1 only induces a mild chlorosis on the inoculated leaves of *N. tabacum* plants. However, the introduction of mutations within the TMV-U1 genome can result in severe yellowing (Dawson *et al.*, 1988). The invasion of plant cells by TMV sometimes results in necrotic symptoms. Necrosis is caused by the virus eliciting the plant's hypersensitive response (Goodman and Novacky, 1994; Section 1.9.1), the aim being to contain the virus by destroying infected cells. While TMV is able to multiply within the cells of these plants they are normally considered to be resistant to the virus. The effectiveness of the response can vary, for example, Holmes (1929) observed that the necrosis induced by TMV on *N. glutinosa* took the form of small lesions. However, the necrosis induced on *N. langsdorffii* and *N. sanderae* was capable of spreading systemically throughout the plants.

1.4 GENERATION OF INFECTIOUS cDNA CLONES

1.4.1 Use of the Enzyme Reverse Transcriptase

Before describing TMV further, it is important to understand some of the techniques which have been applied in order to gain information. Until the early 1980s research carried out on the majority of plant viruses was limited. This was due to the unstable nature of their RNA genomes. Despite this, information concerning the RNAs and proteins associated with TMV infections accumulated. In addition, the 3' end of the TMV-U1 genome was sequenced (Guilley *et al.*, 1979). The limitations imposed by RNA genomes were overcome due to the enzyme reverse transcriptase (reviewed by Boyer and Haenni, 1994). The enzyme facilitates the production of single-stranded cDNAs which can be converted into double-stranded cDNA clones. Using this technology the sequences of TMV-U1 and TMV-L were determined from a range of cDNA clones (Goelet *et al.*, 1982; Ohno *et al.*, 1984). Furthermore, it was possible to generate full length cDNA clones of viral RNA. These could be manipulated using a range of molecular techniques. Investigating the effects of such manipulations presented a problem, as the majority of full length viral cDNA clones were uninfectious to plants. However, there were exceptions, for example i) cDNA clones of potato spindle tubor viroid (PSTV), arranged in tandem, were infectious following the manual inoculation of plants (Cress *et al.*, 1983; Gardner *et al.*, 1986). ii) a cDNA clone of satellite tobacco necrosis virus (STNV) cDNA, flanked by poly dC/dG regions, was infectious when co-inoculated with TNV (van Emmelo *et al.*, 1987). iii) a cDNA clone of alfalfa mosaic virus (AlMV) RNA 3 was infectious if co-inoculated with RNAs 1 and 2 (Dore and Pinck, 1988). None of the described cDNA clones were fused to promoters known to be active in plants and the mechanism behind these infections is not fully understood.

1.4.2 Fusion of cDNA Clones to Promoters Active in Bacteria

To overcome problems with infection full length clones of RNA viruses were fused to bacterial promoters (Boyer and Haenni, 1994). This allowed infectious transcripts to be produced *in vitro* and then manually inoculated on to the leaves of host plants. Ahlquist and Janda (1984) described the successful generation of an infectious cDNA clone of brome mosaic virus (BMV). This was followed by similar reports describing infectious cDNA clones of TMV-U1 (Dawson *et al.*, 1986) and TMV-L (Meshi *et al.*, 1986). When inoculated on to host plants transcripts induced symptoms identical to those induced by purified virus particles. *In vitro* transcripts were originally generated using a modified Pr promoter from the bacteriophage λ, designated Pm.

Investigations were later carried out using bacteriophage SP6 and T7 promoters (Janda *et al.*, 1987). It was thought that they may be superior due to their high initiation rate and improved ability to produce full length transcripts. RNA, transcribed by the T7 promoter was shown to be 50x more infectious to protoplasts and 20x more infectious when inoculated on to plants, compared with RNA transcribed by the Pm promoter (Jander *et al.*, 1987). As a result, many full length cDNA clones of TMV have since have been fused to the T7 promoter (Holt and Beachy, 1991; Chen *et al.*, 1996).

The importance of the fusion, made between the promoter and the 5' end of a viral genome, was recognised by Ahlquist and Janda (1984). As a result they designed a vector which enabled transcription to begin with the first nucleotide of a viral genome. Dawson *et al.* (1986) later demonstrated that the transcription of six additional nucleotides, upstream from the 5' end of the TMV-U1 genome, led to a significant decrease in infectivity. Janda *et al.* (1987) described the infectivity of BMV RNA transcripts containing either zero or one additional 5' nucleotides. In protoplasts, transcripts without an additional 5' nucleotide were shown to be three times as infectious as transcripts with one additional nucleotide. Transcripts of BMV RNA 3, containing either seven or sixteen additional 5' nucleotides were found to have very little biological activity (Jander *et al.*, 1987). Additional non-viral nucleotides at the 3' end of transcripts are tolerated to a greater extent than additional 5' nucleotides (Ahlquist *et al.*, 1984; Dawson *et al.*, 1986; Meshi *et al.*, 1986). However, Dawson *et al.*, (1986) suggested that RNA transcribed from a cDNA clone of TMV-U1 may have been uninfectious due to ~1 000 additional 3' nucleotides. While limiting the number of non-viral 3' nucleotides can increase infectivity, it is considered to be one of the least important criteria for enhancing the infectivity of transcripts (Meshi *et al.*, 1986; Janda *et al.*, 1987). Where transcripts remain infectious, non-viral nucleotides tend to be quickly lost from a viral population (Ahlquist *et al.*, 1984; Dawson *et al.*, 1986; Meshi *et al.*, 1986). It has been suggested that this may be due to *in vivo* modifications or site specific initiation or termination of RNA synthesis (Boyer and Haenni, 1994).

Eukaryotic messenger RNAs (mRNAs) normally have 7-methyl guanosine (M^7GpppG) cap structures at their 5' end. The purpose of these caps is to promote the formation of mRNA-ribosome complexes and to increase the stability of the mRNA (Furuichi *et al.*, 1977). A M^7GpppG cap structure is also present at the 5' end of many viral mRNAs. These include the 5' end of the TMV genome and the 5' end of the subgenomic RNA from which coat protein is expressed (Sections 1.5.1; 1.6.5; 1.6.6). Consequently, infectivity was enhanced if viral transcripts, produced *in vitro*,

also contained a cap structure (Ahlquist *et al.*, 1984; Dawson *et al.*, 1986; Meshi *et al.*, 1986; Janda *et al.*, 1987). Meshi *et al.* (1986) demonstrated that uncapped transcripts, prepared from a cDNA clone of TMV-L, were 10^3-10^4 times less infectious than capped transcripts. In 1956 Gierer and Schramm reported that uncoated TMV RNA was infectious. However, the infectivity was only 0.1 % compared to an equivalent amount of coated RNA. Likewise, the infectivity of viral transcripts was increased if they were reconstituted into virus particles prior to the inoculation of plants (Dawson *et al.*, 1986; Meshi *et al.*, 1986).

1.4.3 Fusion of cDNA Clones to Promoters Active in Plants

To date, most infectious cDNA clones used to investigate TMV have required transcription *in vitro*. However, efforts have also been made to generate clones which can be transcribed *in vivo*. It was hoped that host tissue could be directly infected by such clones using established methods such as the manual inoculation of leaves, the inoculation of protoplasts and the microprojectile bombardment of tissue. It was also anticipated that the clones would be suitable for plant transformation experiments as well as a technique known as agroinfection (Grimsley *et al.*, 1986; Grimsley, 1990). Cauliflower mosaic virus (CaMV) is circular double stranded (ds) DNA virus with a genome of ~8000 base pairs (bp) (Franck *et al.*, 1980). Promoter activity in the nucleotide sequence upstream of the 35S RNA transcript was reported by Guilley *et al.* (1982). This region, termed 35S, has since been cloned and used as a constitutive promoter in plants from both the Cruciferae and Solanaceae families (Odell *et al.*, 1985). A number of viral cDNA clones capable of being transcribed *in planta* have now been reported. All are cloned downstream from the 35S promoter.

When designing 35S driven viral cDNA constructs care was usually taken to ensure that the promoter was directly fused to the cDNA. Failure to observe the importance of the 35S-cDNA fusion may have accounted for the poor infectivity of one of the first reported 35S driven cDNA clones. It consisted of a 35S promoter linked to cDNAs of all three BMV RNAs. Transcripts were produced containing up to twelve additional 5' non-viral nucleotides (Mori *et al.*, 1991). Additional 5' nucleotides were also allowed in 35S driven cDNA clones of beet necrotic yellow vein virus (BNYVV) RNAs 3 and 4 (Commandeur *et al.*, 1991). It is possible that these may have been tolerated, when inoculated on to plants together with authentic RNAs 1 and 2. This is because the viral replicase proteins are translated from BNYVV RNAs 1 and 2. In most cases steps were also taken to limit the number of 3' as well as 5' non-viral nucleotides. This was usually achieved by cloning either transcription termination and polyadenylation signals or a self cleavage ribozyme sequence, downstream from the

viral cDNA. Alternatively restriction enzymes were used to linearise plasmid, at an appropriate site, prior to inoculation. While not essential, reducing the number of non-viral 3' nucleotides has been shown to increase infectivity (Turpen *et al.*, 1993; Dagless *et al.*, 1997). 35S driven cDNA clones of TMV-U1 have been used to infect *N. tabacum* plants via plant transformation (Yamaya *et al.*, 1988) and also agroinfection experiments (Turpen *et al.*, 1993). However, many investigators have concentrated on the ability of 35S driven viral constructs to infect plants via the manual inoculation of leaves. Reports have been made concerning the infectivity of 35S-cDNA clones of pea early browning virus (PEBV) (MacFarlane *et al.*, 1992), TMV-L (Weber *et al.*, 1992), plum pox virus (PPV), (Maiss *et al.*, 1992), cowpea mosaic virus (CPMV) (Dessens and Lomonossoff, 1993), AlMV (Neeleman *et al.*, 1993) cucumber mosaic virus (CMV) (Ding *et al.*, 1995) and zucchini yellow mosaic virus (ZYMV) (Gal-On *et al.*, 1995). The infectivity of these clones, via manual inoculation, was variable. A common observation was that while constructs were capable of infecting some host plants they failed to exhibit the normal host range of the virus (MacFarlane *et al.*, 1992; Weber *et al.*, 1992).

1.5 THE TMV GENOME

1.5.1 Organisation of the Genome

TMV is an obligate parasite, consequently it requires a host in order to provide raw materials, energy and the machinery required for successful replication, translation and movement. Research has revealed that at least four proteins are expressed from genes encoded by the viral genome. With the aid of these proteins TMV is capable of invading and directing the co-operation of host cells. The result is that TMV RNA can become the most abundant nucleic acid species within a plant. The sequences of the entire TMV-U1 and TMV-L genomes have been published (Goelet *et al.*, 1982 and Ohno *et al.*, 1984, respectively). As a result the way in which the genes were organised on a single stranded (+)-sense RNA was revealed (Figure 1.1; Table 1.1). Strong selection pressures must be responsible for maintenance of the small, highly organised TMV genome, which almost entirely consists of gene coding regions. It follows that in order to remain part of this genome each nucleotide must benefit the survival of TMV. Information concerning the way in which TMV infects host plants has been collated over many years. As a result a model for viral replication and gene expression has developed (see Figure 1.1). In order to aid understanding a brief explanation has been provided below and is supported by evidence contained within Sections 1.6 and 1.7. Exact M_r values are quoted for proteins transcribed by TMV-U1 (Goelet *et al.*, 1982).

i) The removal of coat protein from the 5' end of TMV particles facilitates the direct translation of two RNA-dependent RNA polymerase (replicase) proteins, M_r 125 941 (126 kDa) and M_r 183 253 (183 kDa) (Sections 1.6.3; 1.6.5; 1.7.1).

ii) The replicase proteins join with viral RNA and a number of host proteins to form an active replicase complex (Section 1.6.2). This complex recognises sequences at the 3' end of the genomic (+)-sense RNA, resulting in the production of a complementary copy of (-)-sense RNA (Section 1.6.2; 1.6.4).

iii) The replicase complex recognises sequences at the 3' end of the (-)-strand RNA and consequently (+)-sense genomic RNA is produced (Sections 1.6.2; 1.6.3). Replication is associated with the formation of double-stranded RNA species (Section 1.6.2).

iv) The replicase complex also recognises internal subgenomic promoters (SGPs) within (-)-sense RNA, consequently 3' coterminal subgenomic (+)-sense RNAs are produced (Section 1.6.6).

v) A protein, M_r 29 987 (30 kDa), is translated from the 5' end of subgenomic I_2-RNA (Section 1.6.6). This protein facilitates cell-to-cell movement of the virus (Sections 1.7.2).

vi) The coat protein, M_r 17 604 (17.5 kDa), is translated from the 5' end of subgenomic LMC-RNA (Sections 1.6.6). It is capable of coating (+)-sense genomic RNA following an interaction with the origin of assembly (OAS) sequence (Sections 1.6.7; 1.7.3). The coat protein may also facilitate the long distance (systemic) movement of TMV (Section 1.7.3)

vii) It is possible that other proteins are coded by open reading frames (ORFs) within the TMV genome (Sections 1.6.6; 1.6.8). In particular a putative 54 kDa protein may be translated from the 5' end of subgenomic I_1-RNA.

1.5.2 Comparison with Other Viruses

Virologists have observed similarities between groups of viruses which are traditionally considered to be quite distinct. These similarities can be used to discern the way in which viruses evolved (Franssen *et al.*, 1984). In addition the study of one virus can be complemented by the study of related viruses. At the amino acid level the TMV replicase proteins share a degree of homology with the replicase proteins of BMV

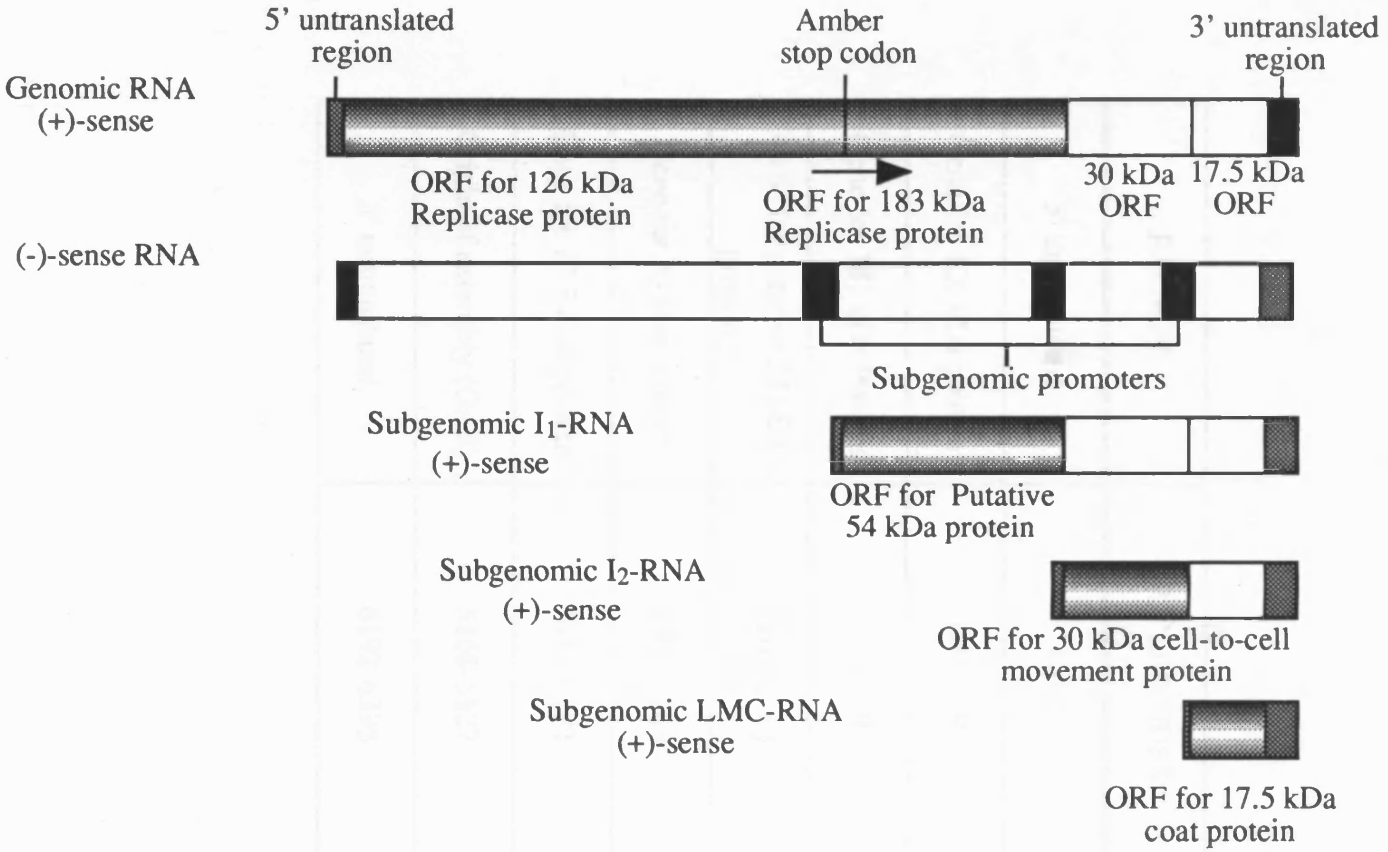


Figure 1.1 Diagram Showing the Organisation of the TMV Genome. The 126 kDa and 183 kDa protein are expressed from the genomic RNA. Other proteins are expressed from 3' coterminal subgenomic RNAs. For accompanying explanation see section 2.5.1 of the text.

Table 1.1 Organisation of the TMV-U1 Genome.
Information is based on the sequence published by Goelet *et al.* (1982).

REGION	NUCLEOTIDES
5' untranslated	1-68
Gene for 126 kDa protein	69-3419
Gene for 183 kDa Protein	69-4919
Gene for putative 54 kDa protein	3495-4919
Gene for 30 kDa protein	4903-5709
Gene for 17.5 kDa protein	5712-6191
Origin of assembly (OAS)	5168-5527
3' untranslated	6192-6395

(*Bromovirus*) and AlMV (Haseloff *et al.*, 1984). These both have a tripartite (+)-sense RNA genome. The replicase proteins are translated from RNAs 1 and 2 whereas the coat protein ORF is situated at the 3' end of RNA 3 and translated from subgenomic RNA 4. The conserved regions within the replicase proteins are also contained within three of the Sindbis virus replicase proteins (Haseloff *et al.*, 1984; Ahlquist *et al.*, 1985). The virus is a (+)-sense RNA *Alphavirus* and infects vertebrates. TMV, BMV and AlMV are now recognised as members of a superfamily of Sindbis-like plant viruses (Matthews, 1991). TMV has also been compared with other animal viruses. It was observed that the genome of poliovirus contained an ORF which coded for a replicase protein. Similarities between the genomes of poliovirus and the Sindbis-like plant viruses led to speculation that the latter also contained genes coding for replicase proteins (Kamer and Argos, 1984). Poliovirus together with foot and mouth disease virus are both members of the *Picornaviridae* family. Some plant viruses, such as CPMV (*Comovirus*) (Franssen *et al.*, 1984), bear a close resemblance *Picornaviridae* and are therefore considered to be members of a Picorna-like superfamily (Matthews, 1991). Finally, it has been shown that the TMV 30 kDa protein resembles proteins produced by plant viruses from other groups. These viruses include tobacco rattle virus (TRV) (Boccara *et al.*, 1986) carnation etch ring virus (CERV) and CaMV (Hull *et al.*, 1986).

1.6 REPLICATION AND GENE EXPRESSION

1.6.1 Site of Viral Replication

The site of TMV replication in infected leaf tissue has been studied by Saito *et al.* (1987b) and Hills *et al.* (1987), using immunocytochemical and immunogold localisation techniques respectively. Saito *et al.* (1987b) reported that the 126 kDa and 183 kDa replicase proteins were localised in granular areas of cytoplasm known as inclusion bodies. These were ovular in shape and had a fine fibrillar structure. Although often found near rough endoplasmic reticulum they were not site specific. It was suggested that the inclusion bodies may be the site of viral replication and generation of subgenomic RNAs (Saito *et al.*, 1987b). The authors remained uncertain about the site at which coat protein was translated and used to assemble RNA into virus particles. However, it was observed that TMV particles occurred in aggregates near but not in the granular inclusion bodies. Hills *et al.* (1987) found that the 126 kDa protein was localised in non-membrane bound areas of the cytoplasm known as viroplasms. Mature viroplasms termed X-bodies are commonly associated with TMV-infected tissue (van Regenmortel, 1981). They are distinct from the inclusion bodies reported by Saito *et al.* (1987b) and made up of endoplasmic reticulum, virus particles and tubules.

Significant amounts of the 126 kDa protein were also detected in pockets at the edge of the viroplasms together with TMV coat protein (Hills *et al.*, 1987). Hills *et al.* (1987) suggested that TMV replication and gene expression may occur in these pockets, the viroplasms being formed after replication has taken place.

The results presented by Saito *et al.* (1987b) and Hills *et al.* (1987) indicate that TMV replicates in the cytoplasm. Greater care was taken by Saito *et al.* (1987b) to ensure that tissue samples contained newly infected cells, where TMV was undergoing active replication. This may in part explain the differences in the results. Encouragingly, Saito *et al.* (1987b) suggested that the granular inclusion bodies and virus aggregates may eventually combine to produce X-bodies. Furthermore, when studying protoplasts Hills *et al.* (1987) detected the 126 kDa protein in amorphous structures within the cytoplasm and did not detect viroplasms. The observations that TMV replication takes place in the cytoplasm are further supported in a report by Okamoto *et al.* (1988). They were only able to detect (-)-sense RNA in extracts prepared from the cytoplasm of TMV infected protoplasts.

1.6.2 Replication of the Viral Genome

RNA-dependent replicase extracts have been isolated from BMV infected tissue (Bujarski *et al.*, 1982; Miller and Hall, 1983). These extracts have been successfully used *in vitro* to study the replication of defined viral RNAs (Miller and Hall, 1983; Miller *et al.*, 1985) and the expression of subgenomic RNA 4 (Miller *et al.*, 1986). Until recently attempts to purify a TMV replicase complex were less successful. Zaitlin *et al.* (1973) reported the partial purification of a membrane bound complex capable of generating full length genomic and (-)-sense TMV RNA *in vitro*. However, while it responded to the addition of TMV RNA it did not demonstrate template specificity. The isolation of crude enzyme complexes only present in TMV-U1 infected tissue or TMV-OM inoculated protoplasts was achieved by Young and Zaitlin (1986) and Watanabe and Okada (1986), respectively. However, only the replication of endogenous-RNA template was observed. A recent report that a membrane-bound TMV replicase complex has been successfully isolated form TMV-L infected tissue is more encouraging (Osman and Buck, 1996). The complex is capable of producing genomic, (-)-sense and subgenomic RNAs. In addition, upon removal of endogenous RNA it has been shown to respond to the addition of TMV-U1 as well as TMV-L RNA.

It has been demonstrated that (-)-strand RNA is produced at a reduced rate compared to genomic RNA (Ishikawa *et al.*, 1991). In addition, while the

accumulation of genomic RNA increases up to 14-18 hours post infection, the majority of (-)-sense RNA is generated within 6 hours. As a result, the limited (-)-sense RNA must be repeatedly used as template for the production of genomic and subgenomic RNA (Section 1.6.6). While the mechanism of replication is not understood, there is little doubt that it occurs via the formation of double-stranded RNAs (dsRNAs). Two types of dsRNA were identified in infected tissue and compared with dsRNAs associated with poliovirus replication (Nilsson-Tillgren, 1970; Jackson *et al.*, 1972). Consequently, they were termed the replicative form (RF) and the replicative intermediate (RI). These dsRNA species are common to infections by viruses with ssRNA genomes and have been the subject of many investigations (Beachy and Zaitlin, 1975; Dawson and Dodds, 1982; Dawson and White, 1979; Young and Zaitlin. 1986; Watanabe and Okada, 1986). While the RF appears to be entirely double-stranded, its purpose in replication is not understood. It was initially thought that the RI had a constant length dsRNA backbone and ssRNA tails of varying length. However, studies of turnip yellow mosaic virus (TYMV) suggest that it is mainly single-stranded and that hybridisation of the strands occurs during the purification procedure (Garnier *et al.*, 1980). The RI is thought to represent progeny (+)-sense RNA annealed to (-)-sense template. There is evidence which suggests that the synthesis of protein parallels dsRNA synthesis (Dawson, 1983). Double-stranded forms of subgenomic RNAs have been reported (Dawson and Dodds, 1982; Osman and Buck, 1996). However, the ability of these RNAs to accumulate by replication, in addition to the mechanism described in Section 1.6.6, remains unclear (Watanabe and Okada, 1986; Ishikawa *et al.*, 1991; Osman and Buck, 1996).

1.6.3 The 5' Untranslated Region

The TMV 5' untranslated region, often referred to as Ω, is well adapted in order to perform several important functions. The 5' untranslated region of TMV-U1 is 68 nucleotides long (Goelet *et al.*, 1982). It shares 78.9 % sequence homology with the equivalent region of TMV-L (Ohno *et al.*, 1984). Both regions contain a M^7GpppG cap structure, a short poly U tract near their 5' terminus and a series of CAA repeats. In addition both lack internal G residues and do not possess any obvious secondary structure. The sequence is likely to play an important role in regulating expression of the TMV encoded 126 kDa and 183 kDa proteins (Section 1.6.5). It has been shown the use of Ω, as a 5' leader sequence, can enhance the efficiency of translation (Gallie *et al.*, 1987a). This may be due to the formation of a disome complex where 80S ribosomes recognise an AUG codon and an AUU codon, at nucleotides 69 and 15 of the TMV-U1 genome respectively (Tyc *et al.*, 1984). The exact mechanism by which translation is enhanced and the significance of the disome complex remains unclear

(Gallie *et al.*, 1988). Viral replication requires that sequences, at the 3' end of (-)-sense RNA, are recognised by the replicase complex. As a result genomic (+)-sense RNA is produced. Studies have revealed that sequences within Ω facilitate viral replication. The analysis of TMV-L mutants led to the observation that nucleotides 2-8 were essential for replication (Takamatsu *et al.*, 1991). Viral replication was also significantly decreased in TMV-L mutants which lacked nucleotides 9-22. It was thought that short deletions, downstream from the essential sequence, may have been tolerated due to the nucleotide repeats present within the region (Takamatsu *et al.*, 1991). Finally, Mundry *et al.* (1991) observed that the lack of G residues in the 5' untranslated region may result in weak bonds forming between the region and the viral coat protein. Upon infection, this may enhance uncoating prior to gene expression and RNA replication (Section 1.6.5).

1.6.4 The 3' Untranslated Region

The 3' untranslated region (UTR) of TMV-U1 is 204 nucleotides long (Guilley *et al*, 1979). Unlike Ω the region has an elaborate three-dimensional structure. Similar structures are present at the 3' end of other *Tobamovirus* genomes as well as the genomes of plant viruses from other groups (Reitveld *et al.*, 1984). Oberg and Philipson (1972) reported that the region appeared to mimic a transfer RNA (tRNA) and could be aminocylated with histidine *in vitro*. It was later reported that the 3' 125 nucleotides could be folded into a tRNA-like structure (Guilley *et al*, 1979). Further analysis revealed that, despite being recognised by tRNA-specific enzymes, it did not resemble a typical tRNA cloverleaf structure. It is now thought that the region forms five pseudoknots (Reitveld *et al.*, 1984; van Belkum *et al.*, 1985). Two of these are associated with the tRNA-like structure and the remaining 3 are situated upstream from the structure. The significance of the three-dimensional structure present at the 3' end of the TMV genome has been investigated by Ishikawa *et al.* (1988) and Takamatsu *et al.* (1990b). Chimeras of TMV-L which had their 3' untranslated region replaced by the equivalent region from TMV-OM, CGMMV or TMV-Cc remained infectious (Ishikawa *et al.*, 1988). However the rate of replication was decreased in chimers containing the 3' untranslated region from either CGMMV or TMV-Cc. It is interesting to note that while TMV-L, TMV-OM and CGMMV can all be aminocylated with histidine, TMV-Cc is instead aminocylated with valine (van Belkum *et al.*, 1985). Takamatsu *et al.* (1990b) prepared mutants of TMV-L which contained deletions in their 3' untranslated regions. Viral RNA containing deletions within the first pseudoknot immediately upstream from the tRNA-like structure (nucleotides 6254-6263 of the TMV-L genome) was incapable of replication. While deletions falling within the region of the next 5' pseudoknot were tolerated, they resulted in a reduced rate of replication.

The pseudoknots appeared to interact with the viral replication complex to initiate (-)-sense RNA synthesis. This process has been described for BMV by Miller *et al.* (1986). Synthesis of the (-)-sense RNA appeared to begin with the penultimate nucleotide (C) at the 3' end of BMV (+)-sense RNAs. It was suggested that the terminal 3' residue (A) was added to (+)-sense RNA by a host coded nucleotidyl transferase enzyme.

1.6.5 Expression of Genes Situated at the 5' End of the Genome

The 126 kDa and 183 kDa replicase proteins are encoded by nucleotides 69-3419 and 69-4919 of the TMV-U1 genome, respectively (Goelet *et al.*, 1982). Due to their situation at the 5' end of the TMV genome they can be directly translated from full length genomic RNA (Knowland *et al.*, 1974; Paterson and Knight, 1975; Bruening *et al.*, 1976; Scalla *et al.*, 1978). The theory of cotranslational disassembly has been proposed in order to explain how RNA is uncoated prior to translation (Wilson, 1984). The theory was developed following the observation that the *in vitro* translation of virus particles could be enhanced 100x if the particles were pretreated at pH 8. It was suggested that the treatment relaxed or removed coat protein subunits at the 5' end of the viral genome and the remaining RNA was disassembled in a progressive manner to enable translation. Following studies *in vitro* , Wilson (1984) identified TMV particle-ribosome complexes which were termed "striposomes". Similar complexes were isolated from tissue at 10-70 minutes post inoculation with TMV (Shaw *et al.*, 1986). The way in which uncoating is triggered *in vivo* has not been fully explained. The mechanism does not appear to be host-specific (Gallie *et al.*, 1987b). It was suggested that, the lack of G residues within the 5' untranslated region may explain weak interactions between the RNA and coat protein subunits (Mundry *et al.*, 1991; Section 1.6.3). Alternatively, uncoating may be enhanced by electrostatic repulsions between the carboxylate groups of coat protein amino acids, aspartic acid and glutamic acid (Culver *et al.*, 1995).

Both the 126 kDa and 183 kDa proteins share a common ATG start codon and downstream sequence. The larger protein is expressed due to readthrough of a "leaky" amber (UAG) stop codon (Pelham, 1978). It is situated at nucleotide 3417 of the TMV-U1 genome (Goelet *et al.*, 1982). The rate of readthrough was reported to be <10 % by Pelham (1978) and 10-20 % by Siegel *et al.* (1978). It has since been suggested that the rate may vary and be dependent on the stage of infection (Dawson and Lehto, 1990). *In vitro* studies revealed that addition of yeast amber suppresser tRNA led to an increase in the rate of readthrough from <10 % to ~70 % (Pelham, 1978). Furthermore, the addition of Mg^{2+} ions or the yeast ochre (UAA) suppresser

tRNA could also increase the rate of readthrough. In 1984, Beire *et al.* reported that two tyrosine tRNAs had been isolated from *N. rustica* which were capable of suppressing the amber stop codon *in vitro.* Other reports have identified tyrosine, glutamine and leucine tRNAs from non-plant sources which are capable of suppressing the codon (Bienz and Kubli, 1981; Valle *et al.*, 1987; Skuzeski *et al.*, 1991). The generation of mutant cDNA clones has been used to study readthrough of the amber stop codon within the 183 kDa protein ORF of TMV-L. If the amber stop codon was replaced by an ochre stop codon viral RNA remained infectious (Ishikawa *et al*, 1986). Both the 126 and 183 kDa proteins were detected following the inoculation of *N. tabacum* plants. Replacement of the amber stop codon with an opal (UGA) stop codon abolished the ability of RNA to infect plants. The sequence surrounding the amber stop codon in both TMV-U1,TMV-L is CAAUAGCAAUUA (Goelet *et al.*, 1982; Ohno *et al.*, 1984). Skuzeski *et al.* (1991) suggested that readthrough may be due to an interaction between this sequence and the 80S ribosome complex. Mutational analysis of this sequence was carried out using the GUS reporter gene. The results showed that the 2 codons downstream from the amber codon played an essential role in enhancing readthrough (Skuzeski *et al.*, 1991).

1.6.6 The Generation of Subgenomic RNAs

Eukaryotic ribosomes normally bind to sites at the 5' end of mRNA. They are then capable of translating the first ORF before breaking the polyribosome complex. Section 1.6.5 describes the translation of two genes which co-exist at the 5' end of the TMV genome. The translation of other TMV encoded proteins from downstream ORFs was harder to explain. A number of RNAs, shorter than genomic length, have been detected in TMV infected tissue. These included a low molecular weight component RNA, designated LMC-RNA, and an intermediate length RNA, designated I_2-RNA. Both are functionally monocistronic and share a common 3' terminal sequence with the full length genomic RNA (Bruening *et al.*, 1976; Beachy and Zaitlin, 1977). Research revealed that the 17.5 kDa coat protein was translated from LMC-RNA (Jackson *et al.*, 1972; Bruening *et al.*, 1976; Higgins *et al.*, 1976; Hunter and Hunt, 1976; Siegel *et al.*, 1976; Beachy and Zaitlin, 1977). It has been shown that LMC-RNA represents nucleotides 5703-6395 of the TMV-U1 genome. The coat protein is translated from nucleotides 5712-6191. This leaves a nine nucleotide, M^7GpppG capped, untranslated leader sequence at the 5' end of LMC-RNA (Guilley *et al.*, 1979). It was demonstrated that the 30 kDa protein was translated from I_2-RNA (Bruening *et al.*, 1976; Beachy and Zaitlin, 1977; Joshi *et al.*, 1983; Ooshika *et al.*, 1984). Following analysis it was suggested that the 5' end of the RNA was uncapped (Joshi *et al.*, 1983). Watanabe *et al.* (1984b) predicted that the 5' end of I_2-RNA was

situated at nucleotide 4838 of the TMV-OM genome. However, Lehto *et al.* (1990) presented evidence suggesting that it was situated at nucleotide 4828 of the almost identical TMV-U1 genome. This would leave a 75 nucleotide 5' untranslated region, upstream of the 30 kDa protein ORF which is situated between nucleotides 4903-5709 of the TMV-U1 genome (Goelet *et al.*, 1982). A number of other RNAs have been associated with TMV infection (Bruening *et al.*, 1976; Beachy and Zaitlin, 1977; Goelet and Karn, 1982; Dawson and Dodds, 1982; Kiberstis *et al.*, 1983 Palukaitis *et al.*, 1983; Morozov *et al.*, 1993). While many of these are thought to be artefacts or breakdown products the intermediate length RNA, designated I_1-RNA, has received attention (Palukaitis *et al.*, 1983; Ooshika *et al.*, 1984). Palukaitis *et al.* (1983) observed that the genomic RNA, LMC-RNA, I_2-RNA and also I_1-RNA were all associated with polyribosomes. Sulzinski *et al.* (1985) suggested that I_1-RNA began with nucleotide 3405 of the TMV-U1 and extended to the 3' terminal. It had a 90 nucleotide long 5' leader sequence upstream of an ORF coded by 3495-4919 of the TMV-U1 genome. When I_1-RNA was translated *in vitro* the product was a 54 kDa protein (Sulzinski *et al.*, 1985). However, this protein has not yet been isolated from TMV infected tissue.

Several ideas were put forward to explain the origin of subgenomic RNAs. They include RNA cleavage, premature termination of (-)-sense RNA synthesis, or internal initiation by the replication complex at sites within (-)-sense RNA. A TMV replicase complex, active *in vitro*, has only recently been isolated (Osman and Buck, 1996; Section 1.6.2). As a result studies using TMV were hindered. However, following studies using BMV, substantial evidence was put forward supporting the theory of internal initiation (Miller *et al.*, 1985). In the presence of a BMV replicase preparation only full length RNA was produced from (+)-sense template. This made the theory of premature termination unlikely. When (-)-sense RNA 3 transcripts were used as template the predominant product was the length of subgenomic RNA 4. Further investigations confirmed that the RNA was identical to subgenomic RNA 4 (from which the coat protein is translated). The BMV encoded replicase proteins are similar to the replicase proteins of other Sindbis-like plant viruses (Section 1.5.2). It was therefore suggested that internal initiation may be a common method for the production of subgenomic RNAs (Miller *et al.*, 1985). It required the replicase complex to recognise specific subgenomic promoter sequences within the (-)-sense RNA. The putative subgenomic promoter within BMV RNA 3 was shown to form a hairpin-loop structure and shared similarities with the untranslated region, situated at the 3' end of the RNA (Miller *et al.*, 1985). Further studies revealed that the promoter was situated immediately upstream from the coat protein start codon and consisted of between 62 and 95 nucleotides (French and Ahlquist, 1988; Marsh *et al.*, 1988).

French and Ahlquist (1988) and Marsh *et al.* (1988) both identified a core sequence which was essential for promoter activity. It was suggested that the replicase complex may assemble at this site (Marsh *et al.*, 1988). A poly A tract was located upstream of the core sequence. Marsh *et al.* (1988) suggested that it may facilitate access to the core sequence. Upstream of the poly A sequence was a series of three almost perfectly repeated motifs. They appeared to have an important role in enhancing the production of subgenomic RNA (French and Ahlquist, 1988; Marsh *et al.*, 1988). French and Ahlquist (1988) observed that if the number of motifs was increased the production of subgenomic RNA also increased. Finally Marsh *et al.* (1988) reported an A/U rich region which separated the core sequence from the coat protein start codon. It was suggested that the region may direct initiation of subgenomic RNA synthesis at the correct site. There are similarities between the BMV coat protein subgenomic promoter and the putative TMV subgenomic promoters. Detailed analysis of these promoter regions has not yet been carried out. Meshi *et al.* (1987) observed that a TMV-L mutant, lacking nucleotides 4936-5604, was capable of expressing coat protein. The 98 nucleotides upstream from the coat protein start codon must therefore have been sufficient to allow generation of the subgenomic LMC-RNA. It has been reported that additional upstream sequences may enhance production of the RNA (Dawson *et al.*, 1989; Dawson and Lehto, 1990).

1.6.7 The Origin of Assembly

TMV RNA is encapsidated bidirectionally from an internal origin of assembly (OAS), or nucleation region (Otsuki *et al.*, 1977; Zimmern, 1977). In addition to encapsidated genomic RNA it is possible to isolate encapsidated subgenomic RNAs from infected tissue. It was observed that encapsidated LMC-RNA could be isolated from tissue infected with TMV-Cc but not TMV-U1 (Bruening *et al.*, 1976; Higgins *et al.*, 1976; Siegel *et al.*, 1976; Beachy and Zaitlin, 1977). This is because the *Tobamovirus* genus can be split into two subgroups depending on the location of the OAS (Meshi *et al.*, 1982b). The first subgroup includes viruses which have the OAS situated within the 30 kDa protein ORF. The second subgroup includes viruses which have the OAS situated within the coat protein ORF. TMV-U1, TMV-OM and TMV-L belong to the first subgroup whereas TMV-Cc and CGMMV belong to the second. In 1977, Otsuki *et al.* reported that the OAS of TMV-OM was contained within ~320 nucleotides. As the OAS was located within a gene coding region Zimmern (1977) suggested that the secondary structure, which included hairpin loops, must be important. The nucleotide sequence for the OAS of TMV-U1 (as well as a number of other strains) was reported by Zimmern in 1983, together with a suggested secondary structure. Three equally spaced hairpin loops, thought to be essential for

encapsidation, were formed incorporating nucleotides 5290-5527 of the TMV-U1 genome. A nine nucleotide long unpaired sequence at the apex of loop 1 (at the 3' end of the OAS) was thought to be particularly important (Zimmern, 1977; Zimmern, 1983). A distinct secondary structure, possibly also part of the OAS, was situated upstream of the loops, nucleotides 5168-5289 of the TMV-U1 genome. Mutant cDNAs were prepared in order to analyse the importance of the hairpin loops. It was shown that the rate of encapsidation was not significantly altered if loops 2 and 3 were deleted from the OAS (Turner and Butler, 1986). Furthermore, a 75 nucleotide sequence thought to be sufficient for encapsidation was identified (Turner *et al.*, 1988). It consisted of nucleotides 5444-5518 of the TMV-U1 genome and included the entire loop 1. The importance of the nine nucleotide sequence, AAGAAGUCG, at the apex of loop 1 was again reported (Turner and Butler, 1986; Turner *et al.*, 1988). In particular, the location of G residues at every third position and the infrequency of C residues was observed. The encapsidation of viral RNA is considered further in Section 1.7.3.

1.6.8 Regulation of Gene Expression

A number of methods have been used to study gene expression, on occasions this has led to conflicting results. It appears that the four TMV encoded proteins, to which functions have been assigned, are individually regulated (the subject has been reviewed by Dawson and Lehto, 1990). The ratio of 126 kDa:183 kDa protein expression has been discussed in Section 1.6.5. Given their function, it was predicted that the replicase proteins should be the first to be expressed at the onset of a TMV infection. However, following investigations in synchronously infected protoplasts it was demonstrated that the 126 kDa, 183 kDa and 17.5 kDa proteins were detected at approximately the same time (Siegel *et al.*, 1978; Watanabe *et al.*, 1984a). This was reported to be between two and seven hours post inoculation. Initially the rate of 126 kDa protein synthesis was highest and reached a peak between 16 and 24 hours post inoculation (Siegel *et al.*, 1978). While synthesis of the replicase proteins continued, the rate of synthesis began to decline and was overtaken by coat protein synthesis. Similar observations were made by Joshi *et al.* (1983), following investigations using intact plants. They reported that accumulation of the 126 kDa protein reached a maximum at 30 hours post infection while accumulation of the coat protein reached a maximum at 37 hours post infection. Moser *et al.* (1988) reported that the synthesis of coat protein reached a maximum between 48 and 71 hours post infection. The level of coat protein then remained constant at 1 mg g^{-1} of tissue. It has been shown that the 30 kDa protein is expressed for a short period at the onset of an infection. Watanabe *et al.* (1984) reported that the protein was expressed in

protoplasts between two and nine hours post inoculation. Other authors suggested that accumulation of the protein continued up to ~24 (Joshi *et al.*, 1983; Tomenius *et al.*, 1987) or even 71 (Moser *et al.*, 1988) hours post infection. Once accumulation had ceased, Moser *et al.* (1988) reported that a constant level of 10-20 ng g^{-1} remained in the cell wall fraction for at least 8 days post infection.

Almost the entire TMV genome consists of gene coding regions. As a result, sequences involved with the regulation of replication and gene expression must be located within functional ORFs. This obviously leads to problems when studying regulation and as a result the mechanisms are poorly understood. Attempts have been made to identify important sequences (Watanabe *et al.*, 1987b; Ogawa *et al.*, 1992). While there are some similarities, the sequences upstream from the different ORFs are quite distinct. Attempts to improve the sequence upstream from the 30 kDa protein were unsuccessful (Lehto and Dawson, 1990; Lehto *et al.*, 1990). However, Lehto *et al.* (1990) did observe that, replacing the I_2-RNA subgenomic promoter sequence with the LMC-RNA sequence altered timing of 30 kDa protein expression. It has been observed that the level of protein expression from subgenomic RNAs may be enhanced if genes are situated close to the 3' end of either the BMV or TMV genome (French and Ahlquist, 1988; Culver *et al.*, 1993). Culver *et al.* (1993) suggested that regulation was at the level of translation as no significant differences in levels of RNA were detected. In contrast, levels of Sindbis virus subgenomic RNAs are higher when promoters are close to the 5' end of the genome (Raju and Huang, 1991). It is possible that as yet unidentified TMV encoded proteins are expressed which may be involved with the regulation of gene expression. It has been suggested the this may be the role of the putative 54 kDa protein (Section 1.6.6; Ogawa *et al.*, 1992). Furthermore, a potential ORF has been identified within the genomes of TMV-U1, TMV-L and TMV-U2. It codes for an ~4 kDa protein (Morozov *et al.*, 1993). Translation of the protein *in vitro* resulted in the formation of an ~54 kDa complex. Polyribosomes bound to the complex in a manner which suggested that it may be a translation factor.

1.7 GENE FUNCTION

1.7.1 The Replicase Proteins

Two large proteins, ~126 kDa and ~183 kDa, were repeatedly associated with TMV infections. While it was originally thought that they may be host encoded and stimulated by TMV infections, investigations revealed they were directly translated from full length viral RNA (Section 1.6.5). Consequently, speculation grew concerning their role as RNA-dependent RNA polymerases. If true, the proteins would

be expected to have ribonucleotide triphosphate (rNTP) binding activity. Following studies of a protein, presumed to be the 126 kDa protein, Evans *et al.* (1985) reported such activity. The results were confirmed following the identification of characteristic NTP binding motifs within the TMV encoded 126 kDa and 183 kDa proteins (Gorbalenya *et al.*, 1989). Further investigations revealed that the 126 kDa protein had guanylyltransferase activity (Dunigan and Zaitlin, 1990). As a result it may be responsible for capping the 5' end of the genomic RNA and the subgenomic LMC-RNA. mRNA is normally capped by a similar enzyme within the cell nucleus.

The requirement of both the 126 kDa and 183 kDa proteins for viral replication was investigated by Ishikawa *et al.* (1986), using mutants prepared from a cDNA clone of TMV-L. Viral transcripts which were capable of expressing the TMV 126 kDa protein but not the 183 kDa protein were uninfectious when inoculated on to plants. Further investigations were conducted using a mutant where the amber stop codon at the 3' end of the 126 kDa ORF had been replaced by a tyrosine codon, so that only the 183 kDa protein was expressed. The yield of mutant virus was 10 % of the yield obtained from TMV-L infected tissue, at 7 days post inoculation. This indicated that, although efficiency was reduced, expression of the 183 kDa protein alone was sufficient for viral replication. Following prolonged infection, the yields from the tyrosine-mutant and TMV-L infected tissue were similar. Sequence analysis revealed that the majority of tyrosine codons had changed to ochre stop codons which allowed readthrough, but surprisingly not amber stop codons (Ishikawa *et al.*, 1986). Studies have since confirmed that viral replication does not occur in the presence of severely deleted 126 kDa and 183 kDa proteins. However, replication can be restored if functional replicase proteins are supplied *in trans* (Ogawa *et al.*, 1991; Ogawa *et al.*, 1992). It has also been shown, via the generation of mutants, that the 30 kDa and the 17.5 kDa proteins are not required for the replication of TMV (Meshi *et al.*, 1987; Takamatsu *et al.*, 1987; Dawson *et al.*, 1988). The TMV replicase proteins have been compared with the replicase proteins of Sindbis virus and the Sindbis-like plant viruses (Section 1.5.2). As a result three distinct domains have been identified. Domain 3 is located within the readthrough portion of the 183 kDa protein. It has be shown to share amino acid sequence similarities with non-structural protein 4 (nsP4) of Sindbis virus (Haseloff *et al.*, 1984). The study of Sindbis virus mutants revealed that nsP4 may have RNA polymerase activity (Hahn *et al.*, 1989). Domain 1 is situated at the amino-terminal of both the 126 kDa and 183 kDa proteins and is similar to Sindbis virus nsP1 (Ahlquist *et al.*, 1985). Results presented by Hahn *et al.* (1989) suggested that the function of nsP1 may be to initiate the synthesis of (-)-sense RNA. Domain 2 is situated at the carboxyl-terminal of the 126 kDa protein and middle portion of the 183 kDa protein, it has been shown to share similarities with Sindbis virus nsP2

(Ahlquist *et al.*, 1985). Evidence suggested that the protein may be involved with the synthesis of subgenomic RNA and possibly the regulation of (-)-sense RNA synthesis (Hahn *et al.*, 1989).

A link between TMV coat protein and the appearance of mosaic symptoms on developing leaves (Section 1.3) cannot be ruled out (Section 1.7.3). However, there is evidence which suggests that the replicase proteins may be responsible for the mosaic by preventing the normal development of chloroplasts (reviewed by Culver *et al.*, 1991). Using *N. sylvestris* it was shown that TMV-U1 mutants, incapable of expressing coat protein, could still induce mosaic symptoms (Culver and Dawson, 1989a). The nucleotide sequence of several attenuated strains of TMV have been compared with more virulent, closely related strains. As a result nucleotide substitutions, thought to be significant, have been identified within the 126 kDa protein ORF (Nishiguchi *et al.*, 1985; Lewandowski and Dawson, 1993). Both authors point out that the accumulation of attenuated strains in host tissue was reduced and that this may explain the mild symptoms.

1.7.2 The Cell-to-Cell Movement Protein

In order for TMV to infect a host plant, RNA must be capable of moving from initially infected cells to neighbouring cells. The 30 kDa protein is located downstream of the replicase genes in the TMV genome (Goelet *et al.*, 1982) and translated from I_2-RNA (Section 1.6.6). Due to its expression at the onset of an infection (Section 1.6.8) it was thought that it may have a role in viral replication (Joshi *et al.*, 1983). However, there is now strong evidence supporting the theory that the 30 kDa protein facilitates cell-to-cell movement of TMV. TMV-LSI is closely related to TMV-L and demonstrates temperature sensitivity. In 1978, Nishiguchi *et al.* reported that TMV-LSI appeared to be incapable of cell-to-cell movement at the restricted temperature of 32°C. Transient experiments in protoplasts demonstrated that replication of the mutant strain was uninhibited at 32°C. Further studies of TMV-LSI were conducted by Leonard and Zaitlin (1982) and Ohno *et al.* (1983). The results suggested that mutations within the 30 kDa protein ORF may be responsible for the temperature sensitive phenotype. In particular a single nucleotide substitution, resulting in amino acid 153 being serine in TMV-LSI as opposed to proline in TMV-L, was identified (Ohno *et al.*, 1983). This mutation was introduced into a cDNA clone of TMV-L (Meshi *et al.*, 1987). As predicted, the synthetic mutant was incapable of cell-to-cell movement at the restricted temperature. TMV-L mutants incapable of expressing the entire 30 kDa protein were able to replicate in protoplasts but did not appear to move throughout *N. tabacum* L cv. Samsun leaves (Meshi *et al.*, 1987). Furthermore, they did not

cause local lesions on the inoculated leaves of *N. tabacum* L cv. Xanthi nc plants (Section 1.9.4).

Following the inoculation of fully developed *N. tabacum* leaves, immunogold cytochemistry revealed that the 30 kDa protein was localised within the plasmodesmata (Tomenius *et al.*, 1987). It reached a maximum concentration at 24 hours post inoculation and appeared to coincide with the start of cell-to-cell movement. These results were supported following further studies of TMV infected plants, as well as transgenic plants expressing the 30 kDa protein in isolation from other TMV proteins (Deom *et al.*, 1990). The protein accumulated throughout the cells of young leaves. However, it was more abundant in older leaves, >12 cm long, especially in the cell wall fraction. Studies have been conducted using the temperature sensitive nitrous acid induced mutant TMV-Ni2519 which is incapable of cell-to-cell movement at 32°C. Like TMV-LSI a specific mutation has been mapped to the 30 kDa protein ORF (Zimmern and Hunter, 1983). It has been demonstrated that cell-to-cell movement of both TMV-Ni2519 and TMV-LSI can occur at 32°C if complemented by strains of TMV capable of movement (Taliansky *et al.*, 1982a and Taliansky *et al.*, 1982b, respectively). Taliansky *et al.* (1982b) demonstrated that TMV-Ni2519 could also be complemented by potato virus X (from the *Potexvirus* genus). Furthermore, it was shown that while TMV was incapable of cell-to-cell movement in *Hordeum vulgare*, movement could be achieved if plants were co-inoculated with TMV and BMV (Taliansky *et al.*, 1982c). It is interesting to note that the movement protein of TMV and BMV are quite distinct (Ahlquist *et al.*, 1985). Complementation studies have also been conducted using transgenic plants (Deom *et al.*, 1987). *N. tabacum* plants expressing the 30 kDa protein were able to complement movement deficient TMV-LSI at 32°C. Further investigations indicated that the complementation of movement deficient mutants was most effective in older leaves (Holt and Beachy 1991). This suggested that a threshold level of the movement protein had to accumulate in the plasmodesmata before cell-to-cell movement could occur.

Recent investigations have revealed important information concerning the way in which the 30 kDa protein may facilitate cell-to-cell movement. Communication between adjoining plant cells occurs via the plasmodesmata, which are made up of narrow channels of cytoplasm. In mesophyll cells the channels have a size exclusion limit (Stokes radius) of ~0.73 nm. This would normally allow the movement of molecules with a molecular weight up to ~800 Da (Wolf *et al.*, 1989). However, it was demonstrated that fluorescent dextrans up to 9.4 kDa could be transported through the plasmodesmata of transgenic plant cells expressing the TMV 30 kDa protein (Wolf *et al.*, 1989; Deom *et al.*, 1990). This movement was most effective in older leaves. It

was judged that the Stokes radius of the plasmodesmata increased to 2.4-3.1 nm. The microinjection of leaf mesophyll cells with the TMV 30 kDa protein and labelled dextrans allowed further studies of cell-to-cell movement (Waigmann *et al.*, 1994). The technique revealed that the 30 kDa protein was capable of increasing the Stokes radius of the plasmodesmata to 3.2-4.3 nm. Consequently, the movement of fluorescent dextrans through the plasmodesmata of both the injected and neighbouring cells was observed. The ability of dextrans to move at five minutes post injection suggested that the pathway induced by the 30 kDa protein was already present in plants, possibly to facilitate the movement of macromolecules (Waigmann *et al.*, 1994). The increase in the Stokes radius of the plasmodesmata, alone, was not sufficient to allow movement of naked RNA. When folded, TMV RNA was judged to have a diameter of 10 nm (Wolf *et al.*, 1989). Citovsky *et al.* (1990) suggested that in addition to signalling enlargement of the plasmodesmata, the 30 kDa protein also guided viral RNA through the plasmodesmata. It was shown that the 30 kDa protein could bind ssRNA in a non-specific manner forming unfolded virus-specific ribonucleoprotein particles (vRNPs) (Citovsky *et al.*, 1990; Citovsky *et al.*, 1992). The complexes were estimated to be <2 nm in diameter and therefore small enough to pass through enlarged plasmodesmata. All genomic RNA except the double-stranded loops, present in the OAS and the 3' untranslated region, was thought to be bound. Five functional domains as well as the amino terminal amino acids are currently though to be important for cell-to-cell movement. Domain A and domain B are coded by the terminal two thirds of the 30 kDa protein ORF. It is thought that they are responsible for binding ssRNA (Citovsky *et al.*, 1992). Domain C is situated closer to the amino-terminal of the protein and is thought to ensure that the protein folds in the correct, elongated manner. Domain D is situated at the carboxyl-terminal and overlaps domain B. Watanabe *et al.* (1992) demonstrated that it can be phosphorylated and suggested that this may enhance movement. Finally, domain E overlaps the carboxyl terminus of domain A and the amino terminus of domain B. It appears to be responsible for increasing the size exclusion limit of the plasmodesmata (Waigmann *et al.*, 1994). Deletions can be tolerated at the carboxyl terminus of the 30 kDa protein but not at the amino-terminal (Gafney *et al.*, 1992; Lapidot *et al.*, 1993). Lapidot *et al.* (1993) suggested that the amino-terminal region may be responsible for the way in which the 30 kDa protein targets the plasmodesmata.

1.7.3 The Coat Protein

The gene coding for the 17.5 kDa coat protein is located at the 3' end of the TMV genome (Guilley *et al.*, 1979; Goelet *et al.*, 1982). It is translated from the 5' end of subgenomic LMC-RNA (Section 1.6.6). The protein is the most abundant TMV

encoded protein (Section 1.6.8) and easily purified from TMV infected tissue. The primary role of the protein is to coat viral RNA and as a result protect it from degradation. It has been shown that the coat protein can exist in different forms (Matthews 1991; Butler *et al.*, 1992). At low pH it forms a helical aggregate which resembles its form around viral RNA. At neutral pH coat protein forms a 20S aggregate consisting of discs made up of 34 coat protein subunits. These are arranged in two layers of 17. Butler and Lomonossoff (1978) explained a theory which suggested that the 20S coat protein discs initiated the encapsidation of viral RNA in a 3' to 5' direction. They were able to support the theory by showing that the incorporation of one disc resulted in ~100 nucleotides being coated (one subunit to three nucleotides). It has since been demonstrated the encapsidation is due to the disc structure of the 20S aggregate and is independent of features within the RNA (Turner *et al.*, 1989). At high pH coat protein exists as a monomer or in small 4S, A-protein, aggregates (Butler *et al.*, 1992). There is evidence to suggest that both may be responsible for coating viral RNA in a 5' to 3' direction (Lomonossoff and Butler, 1980; Turner *et al.*, 1989). The stepwise addition of these small units may explain why encapsidation is slower in the 5' to 3' direction. The timing of encapsidation has been a matter of debate. Lomonossoff and Butler (1980) suggest that coating occurs in both directions simultaneously and is completed at about the same time. However, there is also evidence to suggest that 5' to 3' coating does not begin until 3' to 5' coating is complete (Otsuki *et al.*, 1977; Gaddipati and Siegel, 1990).

The multiplication of TMV occurs predominantly in leaf parenchyma cells. Following passage into the phloem the virus can be passively transported throughout the vascular system via the sieve elements (Atabekov and Dorokhov, 1984). In addition to protection of viral RNA, the coat protein is thought to have an important role in assisting this long distance (systemic) movement. Siegel *et al.* (1962) studied two mutant strains of TMV, one could not produce coat protein while the other could not assemble viral RNA into coated particles. Following the inoculation of the mutants on to *N. tabacum* plants, the slow spread of TMV from leaf-to-leaf was described. Young expanding leaves remained virus free. Further studies, using cDNA clones of TMV-L and TMV-U1, revealed that the systemic movement was severely inhibited if the coat protein ORF was partially or completely deleted (Takamatsu *et al.*, 1987; Dawson *et al.*, 1988). Transgenic *N. tabacum* plants expressing coat protein were later shown to compensate for coat protein defective mutants by increasing the systemic spread of TMV (Holt and Beachy, 1991). Saito *et al.* (1990) demonstrated that, in addition to defective coat protein, mutations introduced within the OAS of TMV-L could also inhibit systemic movement. This suggests an association between the formation of virus particles and systemic movement. It contradicts the previous suggestion that

movement occurs via the formation of virus-specific ribonucleoprotein particles (Dorokhov *et al.*, 1983; Dorokhov *et al.*, 1984; Atabekov and Dorokhov, 1984).

Many reports have suggested that TMV coat protein is involved with symptom formation (reviewed by Culver *et al.*, 1991). There are two types of chlorosis associated with TMV infections (Section 1.3). A link between mosaic symptoms on developing leaves and the accumulation of coat protein in the chloroplasts has been suggested. Coat protein has been found associated with the thylakoids and reported to reduce the activity of photosystem II, which spans the thylakoid membrane (Reinero and Beachy, 1989; Balachandran *et al.*, 1994; Banerjee *et al.*, 1995). However, there is also strong evidence which suggests that mosaic symptoms may be induced by the TMV encoded replicase proteins (Section 1.7.1). The suggestion that the coat protein may induce chlorosis on expanded leaves by altering the organisation of mature chloroplasts is less disputed. The severity of this chlorosis can vary from mild to severe. Siegel *et al.* (1962) observed that both coat protein deficient and assembly deficient strains of TMV induced a yellowing phenotype on inoculated leaves. Jockusch and Jockusch (1968) reported that a yellowing phenotype was associated with the degeneration of chloroplasts. They suggested that the severity of the yellowing may be influenced by the solubility of the coat protein. Yellowing phenotypes have been reported following the inoculation of plants with infectious transcripts containing mutations/deletions in the coat protein ORF (Dawson *et al.*, 1988; Saito *et al.*, 1989; Lindbeck *et al.*, 1991). Dawson *et al.*, (1988) observed that the exact phenotype was dependant on the nature of an individual mutation. Furthermore, a correlation between the accumulation of defective coat protein and the severity of the phenotype was reported. As the mutants were incapable of assembly into virus particles, the yellowing must have been induced by free mutant coat protein or a portion of the uncoated RNA (Dawson *et al.*, 1988). Following the inoculation of plants with infectious transcripts, Lindbeck *et al.* (1991) reported that the majority of either mutant or authentic coat protein remained in the cytoplasm. This is in agreement with results presented by Hills *et al.* (1987; Section 1.6.1). As a result, it was suggested that the deterioration of mature chloroplasts may be due to interactions between coat protein and chloroplast proteins which are synthesised in the cytoplasm.

1.8 TMV-BASED EXPRESSION VECTORS

The use of virus-based vectors for the expression of foreign genes has been investigated in many laboratories, progress has recently been reviewed by Porta and Lomonossoff (1996). The potential of such vectors to replicate autonomously in cells indicates that they may reach higher titres than those achieved by 35S driven non-viral constructs (which are incapable of replication). If infectious vectors are used to

inoculate plants the timing of infection can be regulated. In addition, minor changes can be implemented without the need for repeated plant transformations. However, there are problems associated with inoculating plants with an infectious agent (Joshi *et al.* 1990; Ahlquist and Pacha, 1990). Some of these problems could be addressed if vectors were stably integrated into the genome of host plants. If expressed in every cell of a host plant certain genes, essential for infection but not replication, could be eliminated. It is also possible that an obligate vector-host relationship could be established (Joshi *et al.*, 1990). This would involve disabled expression vectors being inoculated on to transgenic plants which express a *trans*-acting function essential for replication or movement. Such solutions are attractive providing that recombination does not occur. Bujarski and Kaesberg (1986) reported that recombination was possible between mutant and wild type strains of BMV. However, it was not reported to interfere with experiments where mutant viruses were complemented by viral proteins expressed *in trans* (Deom *et al.*, 1987; Holt and Beachy, 1991; Ogawa *et al.*, 1991). Another potential problem concerning the use of viruses as expression vectors may arise due to the lack of proof reading by the viral replicase proteins. In normal circumstances the viral sequence appears to be maintained by strong selection pressures which would not apply to a foreign gene. Van Vloten-Doting *et al.* (1985) predicted a possible error rate of 10^{-4}-10^{-6} per ribonucleotide transcribed and therefore suggested that expression vectors may be of limited use. Siegel (1985) was more optimistic and suggested that in practice the error rate may be less. Kearney *et al.* (1993) studied the stability of foreign sequences within the TMV genome. The error rate was predicted to be $\leq 10^{-4}$ per ribonucleotide for every passage through a host plant. It was concluded that the low level of genetic drift would still allow foreign genes to be efficiently expressed.

A combination of many factors make TMV a good candidate for development as a virus-based expression vector. The virus is capable of multiplying to high titres within host plants (Matthews, 1991; Matthews 1992). As a result of extensive research a knowledge of viral replication and gene expression is developing (Section 1.5; 1.6). The functions of 4 TMV encoded proteins have been determined and the way in which they interact with host factors is being studied (Section 1.7). Furthermore, a number of highly infectious cDNA clones of TMV, capable of being transcribed either *in vitro* or *in vivo*, are available for use (Section 1.4). The expression of small proteins (up to 15 amino acids) by TMV based expression vectors has been relatively successful (Takamatsu *et al.*, 1990a; Hamamoto *et al.*, 1993; Turpen *et al.*, 1995). These have been expressed as fusion proteins, with the foreign sequence incorporated either within or at the 3' end of the coat protein ORF. Hamamoto *et al.* (1993) and Turpen *et al.* (1995) both describe using the leaky stop codon signal, from the 3' end of the TMV

126 kDa protein ORF. The sequence was inserted at the 3' end of the coat protein ORF and allowed the expression of the fusion protein. The yield of vector particles was ~1 mg g^{-1} fresh tissue and similar to the yield of virus particles expected from TMV infected tissue. The coat protein:fusion protein ratio was ~20:1 and corresponded to the rate of readthrough reported by Pelham (1978).

Two methods have been reported which allow the expression of foreign genes up to 1 kb. One approach has been to express a foreign protein in addition to the other proteins encoded by the TMV genome. Alternatively a foreign gene sequence has been used to replace a TMV encoded gene. The first approach was adopted by Dawson *et al.* (1989) in order to express the chloroamphenicol acetyl transferase (CAT) enzyme. Two constructs were built, the least successful contained the ~700 bp CAT gene inserted downstream from the TMV-U1 coat protein ORF. The second construct contained the gene inserted in between the 30 kDa protein ORF and coat protein ORF. This construct was capable of replication, systemic movement and the expression of CAT. However, the vector was unstable and consequently the foreign gene sequence was quickly lost. In order to build the construct the coat protein subgenomic promoter was duplicated and positioned upstream of both the CAT gene and the coat protein gene. It was thought that the problem may have been caused by the repeated subgenomic promoter sequence (Dawson *et al.*, 1989). Donson *et al.* (1991) reported the generation of a new expression vector. A foreign gene was positioned under the control of the TMV-U1 coat protein subgenomic promoter, downstream from the 30 kDa protein ORF. This sequence was followed by the subgenomic promoter and coat protein ORF from odontoglossum ringspot virus (ORSV). The virus is a member of the *Tobamovirus* genus. However, while it contains sequences which are functionally similar to sequences from TMV-U1, its overall sequence homology is low. Using this type of expression vector Donson *et al.* (1991) were able to demonstrate expression of the protein neomycin phosphotransferase (NPT). In addition subgenomic RNAs coding for both the NPT II gene (~800 bp) and the ~200 bp dihydrofolate reductase gene (DHFR) were generated. It was observed that the vectors were not entirely stable following passage through TMV host plants although they were an improvement on the vector described by Dawson *et al.* (1989). A similar vector has since been successfully used to express high levels of the ribosome inactivating α-trichosanthin protein (Kumagai *et al.*, 1993). The expression vector was transcribed *in vitro* prior to the manual inoculation of plants. The foreign 27 kDa protein was estimated to constitute 2 % of the total soluble protein isolated from inoculated plants.

Takamatsu *et al.* (1987) first reported generating a TMV expression vector where the coat protein ORF was replaced by a foreign gene. Two constructs were

described, both contained the CAT gene inserted downstream from the coat protein subgenomic promoter of TMV-L. In one construct the start codon of the CAT gene was inframe with the start codon of the coat protein. In the second construct it was out of frame. Transcripts, prepared *in vitro,* were significantly less infectious than transcripts prepared from a cDNA clone of TMV-L from which the coat protein gene had been deleted. It was suggested that this may be due to toxic effects induced by the CAT protein. Following the inoculation of plants, both constructs expressed low levels of CAT, these were estimated to be ~1 μg g tissue^{-1} (Takamatsu *et al.*, 1987). Expression was lower from the construct containing the CAT gene inserted out of frame with the coat protein start codon. The results presented are similar to those reported by French *et al.* (1986). Using a cDNA clone of BMV, three constructs were generated. Each construct contained the CAT gene inserted in place of the coat protein ORF. Again, the CAT gene appeared to impair viral replication following the manual inoculation of host plants. Expression of CAT was greatest if the foreign gene was inserted inframe with the coat protein start codon. CAT activity was almost halved if the gene was out of frame and separated by a stop codon and reduced to 15 % if not separated by a stop codon (French *et al.*, 1986). Dawson *et al.* (1988) also replaced coat protein gene of TMV-U1 with the CAT gene and detected CAT activity. While leaving the LMC-RNA untranslated leader sequence intact the gene was inserted down stream from a inactivated coat protein start codon. This approach was successfully adopted by Chein *et al.* (1996). Using full length cDNA clones of TMV-U1 and the rakkyo strain of TMV (TMV-R), the coat protein ORFs were replaced with an ~800 bp gene coding for a green fluorescent protein (GFP). In each case, the GFP gene was inserted ten nucleotides downstream from an inactivated coat protein start codon. The authentic 3' untranslated regions remained, together with the 3' ends of the coat protein ORFs. Transcripts were generated *in vitro* and inoculated on to the leaves of *N. tabacum* cv. Bright yellow plants. TMV-U1 is capable of systemically infecting *N. tabacum* cv. Bright yellow, while TMV-R is confined to the inoculated leaves (Chein *et al.*, 1996). Transcripts generated from the TMV-U1-GFP construct induced a strong fluorescence on inoculated leaves. The fluorescence was first detected at 3 days post inoculation and was observed for ~4 weeks. Only faint fluorescence was observed on leaves inoculated with transcripts generated from the TMV-R-GFP construct. Finally, replacement of the 30 kDa protein ORF with the GUS gene (~1.8 kb) has been reported (Lapidot *et al.*, 1993). While transcripts, generated *in vitro*, were incapable of cell-to-cell movement they did express GUS in directly inoculated cells. Furthermore, if inoculated on to transgenic plants expressing the 30 kDa protein cell-to-cell movement, accompanied by GUS expression, was observed.

1.9 RESISTANCE TO TMV

1.9.1 Introduction to Crop Protection

Many measures can be applied to reduce the risk of infecting plants with TMV. These include the sterilisation of soil, surface sterilisation of seeds, careful disposal of plant debris and the thorough cleansing of overalls and tools. If such measures are either unfeasible or not strictly adhered to infections can occur. As a result, the use of resistant plants is an attractive proposition. The definition of resistance can be confusing and has been discussed by Matthews (1991). The use of the term "resistance" within this thesis refers to the limitation of disease development in a plant species normally considered to be a systemic host of a particular pathogen (in most cases this is TMV). For several decades plant breeders have attempted to identify and exploit naturally occurring genes which confer resistance to TMV. Unfortunately the use of such genes has often been accompanied by the isolation of resistance breaking strains. Consequently, Pelham (1972) warned that resistance should be "the last line of defence in an integrated programme of control". Many of the experiments described within this thesis were aimed at understanding the way in which TMV interacts with the *Tm-1* and *N* resistance genes. These genes will be discussed further within this introduction (Sections 1.9.2-1.9.5) together with the *Tm-2* gene, its allele the $Tm\text{-}2^2$ gene, and the *N'* gene.

The hypersensitive response (HR) is a common response in plants following pathogen attack. Induction of the HR by viruses has been reviewed by Goodman and Novacky (1994). Typically, the HR results in the collapse of cells at and surrounding a site of infection and as a result, disease can be contained. Cell death can normally be monitored by the appearance of necrotic lesions. The induction of soluble pathogen related (PR) protein expression is associated with the HR (van Loon, 1985). Evidence suggests that they are induced by an increase in the level of salicylic acid throughout the plant (Ohshima *et al.*, 1990; Yalpani *et al.*, 1991; Uknes *et al.*, 1993). The PR proteins are classed as either acidic or basic. Following the inoculation of *N. tabacum* cv. Samsun *NN* plants with TMV, Dore *et al.* (1991) observed that acidic proteins were localised in extracellular spaces while basic proteins accumulated within cell vacuoles. The distribution of the PR proteins in TMV infected *N. tabacum* cv. Samsun *NN* plants has been examined by Heitz *et al.* (1994). The level of all PR proteins was highest in the tissue immediately surrounding lesions. Very low levels of acidic PR proeins were detected in healthy tissue which remained, between lesions, on inoculated leaves. Elevated levels of basic PR proteins were detected throughout inoculated leaves. The PR proteins were detected in varying amounts in upper systemically

infected leaves. It is thought that the systemic expression of the PR proteins may account for systemic acquired resistance (Yalpani *et al.*, 1991; Uknes *et al.*, 1993). This is where symptom formation is reduced following secondary infections by the same or a different pathogen. Other events which accompany the HR include electrolyte leakage from cells, lignification of cell walls and an increase in destructive oxygen-centred free radicals (Goodman and Novacky, 1994). As a result of infections by viruses, the HR appears to be dependent on intercellular communications and does not occur in isolated systems such as protoplasts (Goodman and Novacky, 1994).

Cross protection, where one strain of a virus offers protection against the invasion of a second strain, was described by McKinney, (1929). It has since been the subject of many investigations and a number of theories have been proposed to explain the phenomenon. These include competition for either essential metabolites or sites of viral multiplication, encapsidation of RNA by excess coat protein before translation can occur, or the expression of some form of protecting agent (Matthews, 1991). Difficulties were encountered when attempting to validate these theories. Recently progress has been made due to the ability to transform plants with cDNA clones of specific viral sequences. This allows any resistance induced by the sequences to be studied in isolation from the complete viral genome and associated proteins. The results suggest that cross protection may be effected by a number of different mechanisms (Section 1.9.6). It is possible that these can be exploited for disease control.

1.9.2 The *Tm-1* Gene

In 1956, Walter reported an inheritable form of resistance to TMV which was thought to originate from *L. hirsutum*. The resistance was described as nonsymptomatic tolerance due to the observation that inoculated plants remained symptomless but still permitted the replication and spread of TMV. Further investigations were conducted following successful transfer of the gene, designated *Tm-1*, into *L. esculentum* (Clayberg *et al.*, 1960). Levesque *et al.* (1990) suggested that the *Tm-1* gene was situated on the top arm of chromosome 2 and to date this remains undisputed (Tanksley *et al.*, 1992; Ohmori *et al.*, 1995). The gene has been linked to a repetitive ribosomal DNA (rDNA) sequence which is also thought to originate form *L. hirsutum* (Levesque *et al.*, 1990). In addition to the rDNA marker, which was identified by restriction fragment length polymorphism (RFLP), a number of random amplified polymorphic DNA (RAPD) markers have been linked to the *Tm-1* gene (Ohmori *et al.*, 1995).

Clayberg, (1960) and Pelham, (1972) reported that the multiplication of TMV was permitted in a *Tm-1* background. Furthermore, the virus accumulated at higher levels in plants heterozygous for the gene compared with plants homozygous for the gene. Both authors reported that it was common for symptom formation to be suppressed in plants heterozygous for the *Tm-1* gene. Fraser and Loughlin (1980) compared the ability of an Ohio isolate, TMV-O, to multiply in *L. esculentum* plants homozygous for the gene (*Tm-1/Tm-1*), plants heterozygous for the gene (*Tm-1*/+) and plants which did not possess the gene (+/+). They observed that the gene was dominant for the suppression of symptoms formation but only partially dominant for the suppression of virus accumulation. It was demonstrated that viral multiplication was inhibited by 90-95 % in homozygous plants and by 70-75 % in heterozygous plants. The inhibition was observed in both the inoculated and systemically infected leaves. Virus accumulation was inhibited to approximately the same extent if plants were inoculated with TMV-U1 (Fraser and Loughlin, 1980). The inhibition of TMV multiplication has also been observed in protoplasts isolated from *L. esculentum* plants containing the *Tm-1* gene (Motoyoshi and Oshima, 1977; Motoyoshi and Oshima, 1979, Watanabe *et al.*, 1987a). Watanabe *et al.* (1987a) reported that inhibition of TMV-L multiplication in protoplasts prepared from plants homozygous for the *Tm-1* gene was similar to that reported by Fraser and Loughlin (1980). The effect of the *Tm-1* gene is dependent on the challenging strain of TMV (Pelham, 1972; Fraser and Loughlin, 1980, Fraser *et al.*, 1980)). In particular, crop breeders became increasingly concerned about a number of strains of TMV which cause disease on *L. esculentum* plants homozygous for the *Tm-1* gene.

Fraser and Loughlin (1980) observed that the multiplication of TMV-O was comparable in plants grown at 33°C irrespective of whether the *Tm-1* gene was present. However, the formation of symptoms was still suppressed. This suggested that symptom formation and viral multiplication were influenced by two separate mechanisms. The ability to detect TMV in the systemically infected leaves of plants expressing the *Tm-1* gene indicated that, despite being at a reduced level, replication and the expression of proteins from subgenomic RNAs was still possible. As a result, Watanabe *et al.* (1987a) suggested that the *Tm-1* gene may be activated at the onset of an infection and interfere with function of the 126 kDa and 183 kDa replicase proteins. Meshi *et al.*, (1988) compared the nucleotide sequence of TMV-L with a related resistance breaking isolate, TMV-Ltal. They revealed that the ability to overcome the *Tm-1* resistance gene was conferred by two nucleotide substitutions within the ORFs for the replicase proteins. These were effectively transferred into an infectious cDNA clone of TMV-L. At nucleotide 3006, C was replaced by G resulting in the amino acid glutamine becoming glutamic acid and at nucleotide 3021, C was replaced by U

resulting in histidine becoming tyrosine. These alterations were also identified when resistant breaking isolates TMV-L_{11}Y237 and TMV-CH2 were compared with TMV-L (Meshi *et al,* 1988). Further TMV-L mutants were prepared which contained just one of the nucleotide substitutions (Meshi *et al.*, 1988). It was demonstrated that the substitution at nucleotide 3006 was sufficient to overcome the *Tm-1* resistance gene. However, sequence analysis revealed that progeny RNA commonly contained the second substitution at 3021. The amino acid substitutions were within domain 2 of the viral replicase proteins (Section 1.7.1). Meshi *et al.* (1988) observed that both the substitutions decreased the local net charge of the replicase proteins. They therefore suggested that there may be an electrostatic interaction between the *Tm-1* gene and the proteins. Strains of TMV not associated with resistant breaking progeny, including TMV-U1, had a higher net charge in the region of the substitutions. Two hypotheses have been proposed which may explain activity of the *Tm-1* gene (Meshi *et al.*, 1988). The first was that the gene, or a related resistance factor, may inactivate the replication machinery. If this was true resistant breaking strains may be able to evade attack or have the ability to disable the resistance factor. Results presented by Fraser and Loughlin (1980) indicated that inactivation did not appear to be due to the degradation of viral RNA. The second hypothesis was that the *Tm-1* gene may be an altered host factor which could not always function in the manner required for viral replication.

1.9.3 The *Tm-2* and *Tm-2²* Genes

In addition to the *Tm-1* gene, two other genes conferring resistance to TMV have been transferred to *L. esculentum*. They are alleles which originate from *L. peruvianum* and have been designated *Tm-2* and *Tm-2²* (discussed by Alexander, 1971). Studies have revealed that the genes are situated at a locus on chromosome 9 of the *L. esculentum* genome (Khush and Rick, 1964; Young *et al.*, 1988). While the *Tm-2* and *Tm-2²* genes are normally considered to be dominant for resistance their exact response to TMV depends on a number of factors. These include whether plants are homozygous or heterozygous for the genes, the invasive strain of TMV and the temperature at which plants are kept before and during infection (reviewed by Hall, 1980). Under certain conditions plants expressing the genes can develop necrosis following inoculation with TMV. This can be accompanied by a suppression of viral movement and is therefore considered to be a hypersensitive response (Section 1.9.1). In some cases, especially in plants expressing the *Tm-2²* gene, the necrosis can be systemic and accompanied by leaf distortion and stunting. Both the *Tm-2* and the *Tm-2²* genes have been associated with resistance breaking strains of TMV. However, strains capable of overcoming resistance conferred by the *Tm-2²* gene are less common. Furthermore, by overcoming the resistance they normally induced easily

distinguishable necrotic/distorted symptoms. Consequently, the *Tm-2²* gene has been regarded as the most useful for crop protection (Hall, 1980; Weber *et al.*, 1993).

TMV is capable of replicating in protoplasts prepared from *L. esculentum* plants possessing either the *Tm-2* or *Tm-2²* genes (Motoyoshi and Oshima 1975; Motoyoshi and Oshima, 1977). This is consistent with the theory that TMV is capable of inducing a *Tm-2/Tm-2²* gene-mediated HR (Goodman and Novacky, 1994). However, replication in protoplasts would also be expected if the genes altered the ability of TMV to move from cell-to-cell (Section 1.7.2). The sequence of a number of resistance breaking tomato strains of TMV have been compared with the sequence of TMV-L. In each case nucleotide substitutions within the 30 kDa cell-to-cell movement protein ORF, resulting in amino acid substitutions, were identified. The majority altered the local net charge of the protein. Three strains capable of overcoming the *Tm-2* gene (TMV-Ltb 1, TMV-C32 and TMV-L11) contained lysine at amino acid residue 133 (Meshi *et al.*, 1989; Calder and Palukaitis, 1992). The equivalent residue encoded by the TMV-L genome is glutamic acid. The substitution was within domains A and E of the 30 kDa protein (Section 1.7.2). All three strains capable of overcoming the *Tm-2* gene contained addition substitutions at varying positions within the 30 kDa protein ORF. Using a synthetic mutant, Meshi *et al.* (1989) demonstrated that the single amino acid substitution at residue 133 was not sufficient to confer the resistance breaking phenotype. A number of amino acid substitutions have been identified within the 30 kDa protein of two strains of TMV (TMV-L11A and ToMV-2²) which are capable of overcoming the *Tm-2²* gene (Calder and Palukaitis, 1992; Weber *et al.*, 1993). Weber *et al.* (1993) demonstrated that two of these substitutions were responsible for conferring the resistance breaking phenotype. In order to demonstrate this the substitutions were introduced into the genome of TMV-L. As a result, amino acid 238 was arginine instead of serine and amino acid 244 was glutamic acid instead of lysine. The substitutions were within domains B and D of the 30 kDa protein (Section 1.7.2). Both are situated at the carboxyl terminus of the 30 kDa protein and are not considered to be essential for cell to cell movement (Gafney *et al.*, 1992). Further investigations are required in order to understand the way in which the *Tm-2* and *Tm-2²* may interact with the TMV cell-to-cell movement protein and/or induce the HR.

1.9.4 The *N* Gene

Maybe the most well known gene conferring resistance to TMV is the *N* gene of *N. glutinosa* (Holmes 1929). Having realised its potential for crop protection it was transferred into *N. tabacum* (Holmes, 1938). The dominant *N* gene confers resistance by inducing the plants' HR (Section 1.9.1). Defined lesions appear on the inoculated

leaves of plants expressing the gene at 2-3 days post inoculation. They continue to expand up to 8-10 days post inoculation and TMV can be detected in tissue within and immediately surrounding the lesions (Deom *et al.*, 1991). Otsuki *et al.* (1972) studied the effect of TMV on *N. tabacum* cv. Samsun *NN* plants (possessing 2 copies of the *N* gene) and *N. tabacum* cv. Samsun *nn* plants. The virus was able to multiply and spread at the same rate in both plants up to the point when lesions first appeared. Further studies demonstrated that accumulation of the cell-to-cell movement protein and the coat protein was also normal up to the point when lesions appeared (Moser *et al.*, 1988). The synthesis of both proteins then ceased and levels of the 30 kDa protein declined until it was undetectable within the cell wall fraction. The *N* gene demonstrates reversible temperature sensitivity. As a result TMV was capable of systemically infecting plants, without the development of necrosis, if they were kept at temperatures above 28°C (Otsuki *et al.*, 1972). TMV is able to multiply normally in protoplasts prepared from plants expressing the *N* gene (Otsuki *et al.*, 1972).

It has been shown that TMV mutants which could not express coat protein were still able to induce the *N* gene mediated HR (Takamatsu *et al.*, 1987; Dawson *et al.*, 1988; Holt and Beachy, 1991). TMV mutants which were incapable of expressing the cell-to-cell movement protein did not induce visible lesions when inoculated on to host plants expressing the *N* gene (Meshi *et al.*, 1988; Holt and Beachy, 1991). Virus must have been confined to initially inoculated cells. It is therefore possible that the death of these single cells remained undetected. Alternatively, it is possible that the cell-to-cell movement of either TMV RNA or TMV encoded protein is required to elicit the *N* gene. Deom *et al.* (1991), demonstrated that at 24°C, movement of a 9.4 kDa dextran was possible in transgenic *N. tabacum* cv. Xanthi plants expressing the 30 kDa protein (Section 1.7.2). However the 9.4 kDa dextran was incapable of movement in similar transgenic *N. tabacum* cv. Xanthi nc plants (expressing two copies of the *N* gene). The results indicated that cell-to-cell movement was possible, yet restricted in the presence of the *N* gene. Movement of the 9.4 kDa dextran was unrestricted in plants kept at 33°C. Deom *et al.* (1991) suggested that, while the 30 kDa cell-to-cell movement protein did not induce the HR, it may have a role in induction or maintenance. To date only one strain of TMV, designated TMV-Ob, has been identified which is capable of systemically infecting plants expressing the *N* gene (Tóbiás *et al.*, 1982). Chemical mutagenesis was performed on TMV-Ob and a mutant, designated TMV-ObNL-1, was selected which could no longer overcome the resistance gene (Padgett and Beachy, 1993). A single nucleotide substitution responsible for the resistant phenotype was identified within the coding region for the 126 kDa and 183 kDa proteins. As a result amino acid 1089 was proline in TMV-Ob and leucine in the mutant TMV-ObNL-1. The substitution did not alter the

local net charge of the protein (Padgett and Beachy, 1993). It is interesting to note that both TMV-U1 and TMV-L contain a proline at amino acid 1089.

The temperature sensitivity of the *N* gene has recently aided its cloning via insertional mutagenesis (Whitham *et al.*, 1994; Dinesh-Kumar *et al.*, 1995). The transposable *Ac* element was used to interrupt the *N* gene in transgenic *N. tabacum* cv. Samsun *NN* plants. F1 generation seedlings, heterozygous for the *N* gene, were then grown at 30°C and inoculated with TMV. Once systemic infections had established the seedlings were transferred to 21°C. If seedlings remained healthy, it was presumed that the *Ac* element had inserted into the coding region for the *N* gene and as a result inactivated it. The identity of a 10.6 kb fragment containing the *N* gene was confirmed following its transformation into *N. tabacum* SR1 plants (Whitham *et al.*, 1994). It has since been shown that the *N* gene is active in transgenic *L. esculentum* plants (Whitham *et al.*, 1996). The gene has been fully sequenced and appears to encode a 131.4 kDa protein and a truncated 75.3 kDa protein (Whitham *et al.*, 1994; Dinesh-Kumar *et al.*, 1995). It was suggested that the latter was generated by alternative splicing of exons designated AE1 and AE2. Sequence analysis identified three regions of interest i) a nucleotide binding domain similar to those found in kinases, ATPases and Ras proteins, in particular it contained motifs known to bind ATP and GTP; ii) a leucine-rich region consisting of 14 imperfect tandem repeats, these regions could be important for protein-protein interactions, cell adhesion and membrane associated functions; iii) an amino-terminal domain bearing similarities to cytoplasmic regions of the *Drosophila* Toll protein and the mammalian interleukin-1 receptor, both trigger intracellular signal transduction cascades by activating transcription factors. While theories are being developed, the way in which the TMV interacts with the *N* gene to elicit the HR is not yet fully understood.

1.9.5 The *N'* gene

The *N'* gene was identified in *N. Sylvestris* (Valleau and Johnson, 1942) and confers resistance to some strains of TMV by inducing the HR. TMV-U1 and TMV-OM are both capable of systemically infecting plants which express the *N'* gene. However the gene is effective against TMV-L and as a result the virus is confined to necrotic lesions. Fraser (1983) reported that the *N'* gene demonstrated temperature sensitivity. In addition, lesion size and TMV multiplication was greater in plants expressing the *N'* gene than in plants expressing the *N* gene. There is substantial evidence indicating that TMV coat protein is the elicitor of *N'* gene mediated HR. Chemically induced mutants of TMV-U1 have been described which induce the HR in *N. sylvestris* (Tsugita and Frankel-Conrat, 1960). Following analysis, mutations

were localised to the carboxyl terminus of the coat protein. Further to this, Fraser (1983) demonstrated a correlation between the thermal stability of viral coat protein and the ability of TMV to induce lesions on plants expressing the *N'* gene. Chimeric hybrids of TMV-OM have been prepared which contain the coat protein gene from TMV-L. Hybrid transcripts, generated *in vitro*, were able to induce necrosis when inoculated on to *N. sylvestris* and *N. tabacum L.* cv Bright Yellow plants expressing the *N'* gene (Saito *et al.*, 1987a). Further experiments led to the identification of sequences within the TMV-L coat protein gene which appeared to be responsible for the proteins ability to elicit the HR (Saito *et al.*, 1987a; Saito *et al.*, 1989). Studies by Knorr and Dawson (1988) and Culver and Dawson, (1989b) identified a number of amino acid substitutions within the coat protein of TMV-U1, which allowed the protein to elicit the *N'* gene-mediated HR. Culver *et al.* (1994) suggested a weak quaternary, but not tertiary, coat protein structure may promote the access of a resistance factor to specific binding sites.

Evidence demonstrating that coat protein alone was able to elicit the *N'* gene, was obtained following plant transformation experiments (Culver and Dawson, 1991; Pfitzner and Pfitzner, 1992). In both cases *Agrobacterium tumefaciens* was used to transform plants with 35S-coat protein constructs. Culver and Dawson (1991) transformed *N. sylvestris* plants with either the coat protein gene from TMV-U1 or the equivalent gene from mutant strains capable of eliciting the *N'* gene. Callus transformed with non-elicitor TMV-U1 coat protein regenerated into healthy plants. However callus transformed with elicitor coat protein regenerated slowly. While necrosis tended to be absent in callus and young plants, it developed as the plants grew and in some cases led to the collapse of entire leaves. Culver and Dawson (1991) suggested that the delay in necrosis may indicate developmental regulation of *N'* gene mediated resistance. They also observed that the development of necrosis appeared to be influenced by the strength of an elicitor coat protein and not the concentration of protein. Pfitzner and Pfitzner (1992) transformed the coat protein gene from TMV-L into *N. tabacum* cv. Samsun and near isogenic *N. tabacum* cv. Samsun EN (which expressed the *N'* gene) plants. At four weeks post co-cultivation callus formation on *N. tabacum* cv. Samsun EN explant was ~10 % of that on *N. tabacum* cv. Samsun explants. Furthermore the callus on Samsun EN explants developed dark brown necrosis by nine weeks post co-cultivation, and in some cases completely died. Both types of transgenic callus expressed similar levels of coat protein (Pfitzner and Pfitzner, 1992). In addition, while PR-1 proteins were detected in both types of callus levels were higher in callus containing the *N'* gene.

1.9.6 Resistance Conferred by Sequences from the TMV Genome

As described in Section 1.9.1, plants infected by a virus can be protected against secondary infections, possibly by more severe strains. However, there are ethical and practical problems associated with the deliberate infection of plants even with a mild pathogen. Such problems may be overcome if transgenic plants express only limited viral sequences, yet sufficient to confer the protection. While there are many theories, the mechanisms of cross protection are not understood. It was thought that further understanding may be gained by studying the protection afforded to transgenic plants expressing part of a viral genome. Transgenic plants have been generated in a number of laboratories using sequences derived from a range of plant viruses. As a result, protection induced by a number of apparently different mechanisms has been reported. Where a virus consists of an RNA genome it can be assumed that protection is afforded by the RNA, viral proteins expressed from the RNA or in some cases truncated/mutated viral proteins. Detailed studies of the transgenic plant are required to further understanding and as a result exploit the different mechanisms of protection.

In 1986, Powell Abel *et al.* reported that a 35S-coat protein construct prepared from a cDNA clone of TMV-U1 had been integrated into the genome of *N. tabacum* cv. Xanthi plants. Following manual inoculation of the transgenic plants with purified TMV-U1 particles symptoms were either absent or delayed. This resistance was later confirmed following the manual inoculation of plants with either TMV-U1 particles or particles of the more severe strain TMV-PV230 (Nelson *et al.*, 1987). Furthermore, the accumulation of virus in inoculated leaves as well as the first infected systemic leaf was reduced. Register and Beachy (1988) observed that a degree of resistance was also active in leaf mesophyll cell protoplasts prepared from the transgenic plants. Both Nelson *et al.* (1987) and Register and Beachy (1988) reported that resistance was overcome if plants were inoculated with TMV RNA as opposed to virus particles. In addition the resistance was overcome if purified TMV particles were pretreated at pH 8 prior to the inoculation of plants (Register and Beachy, 1988). It has been reported that treatment at pH 8 relaxes the 5' coat protein sub-units and aids the onset of an infection (Section 1.6.5; Wilson, 1984). A similar observation was made by Sherwood and Fulton (1882) when studying classical cross protection. They showed that light green, TMV infected tissue (Section 1.3) could be superinfected by a second stain if inoculated with uncoated viral RNA. Wisniwiski *et al.* (1990) demonstrated that the resistance conferred by TMV coat protein in transgenic plants appeared to be greater in systemically infected leaves. Furthermore, the systemic resistance was not overcome by inoculating plants with viral RNA. It was suggested that expression of

coat protein inhibited the movement of virus through the plants vascular system (Section 1.7.3). This was supported by the observation that the rate of TMV movement from wild type root stock to similar upper leaves was reduced if it passed through transgenic stem tissue expressing TMV coat protein (Wisniwiski *et al.*, 1990). Reimann-Phillip and Beachy (1993) reported that a degree of resistance to TMV was observed if the coat protein gene was expressed only in the upper epidermis and xylem tissue of transgenic *N. tabacum* plants. However, the resistance was more effective if coat protein was constitutively expressed in all tissue types. Expression of coat protein in the phloem alone did not confer resistance to TMV (Reimann-Phillip and Beach, 1993). From the available evidence, it appears that there may be more that one mechanism of coat protein-mediated protection. The resistance demonstrated against TMV particles may be effective at an early stage of infection. For example, the cellular uptake or uncoating of virus particles may be inhibited. Resistance to the systemic spread of TMV may be due to a second mechanism where endogenous coat protein inhibits long distance movement.

Donson *et al.* (1993) attempted to transform *N. tabacum* cv. Xanthi plants with two different TMV replicase gene constructs. The first contained nucleotides 1-5085 of the TMV-U1 genome, the second contained nucleotides 1-5463. Following the regeneration of transgenic plants, only plants expressing the second construct demonstrated resistance to TMV. Analysis of the resistant plants revealed that a transposable element with four upstream stop codons had been inserted at nucleotide 2875 of the TMV-U1 genome (Donson *et al.*, 1993). It was concluded that, the production of a single truncated replicase protein was responsible for conferring the resistance. Further analysis revealed that the resistance was effective against a broad range of tobamoviruses These included TMV-U1, TMV-L, TMV-U5, tobacco mild green mosaic virus (TMGMV), green tomato atypical mosaic virus (GTAMV) and ribgrass mosaic virus (RMV). Replication was greatly reduced in inoculated leaves and almost no virus was detected in upper leaves. It was suggested that the accumulation of virus in inoculated leaves may have been below a threshold level required for systemic transport (Donson *et al.*, 1993). The putative 54 kDa protein is encoded by an ORF which overlaps the 3' terminal of the 183 kDa protein ORF (Section 1.6.6). A 35S-cDNA clone of the 54 kDa protein ORF from TMV-U1 has been integrated into *N. tabacum* cv. Xanthi plants (Golemboski *et al.*, 1990). It was reported that the transgenic plants displayed resistance to TMV. As a result, following manual inoculation with either virus particles or RNA, symptoms did not appear and viral replication was significantly inhibited. Carr and Zaitlin (1991) confirmed these observations and reported that the resistance was also effective in isolated protoplasts. Surprisingly, despite being transcribed by the 35S promoter the 54 kDa protein was not

detected in the transgenic plants (Golemboski *et al.*, 1990; Carr *et al.*, 1992). The co-transfection of *N. tabacum* protoplasts with TMV-U1 particles and a cDNA construct capable of expressing the TMV-U1 54 kDa protein resulted in the inhibition of viral replication (Carr *et al.*, 1992). When mutated 54 kDa cDNA constructs which could not express the protein were used resistance was not observed. This provides only indirect evidence that expression of the 54 kDa protein is required to confer the resistant phenotype. It has been suggested that the 54 kDa protein is involved with the regulation of replication (Ogawa *et al.*, 1992). The level of replication may therefore be altered if transgenic plants are already expressing the protein prior to the onset of an infection. When *N. benthamiana* plants were transformed with a 35S-clone of the 54 kDa protein ORF from pepper mild mottle *Tobamovirus* strain S (PMMV-S) two types of resistant phenotype were observed (Tenllado *et al.*, 1995). The first was similar to the resistance described by Golemboski *et al.* (1990) and Carr and Zaitlin (1991). The second phenotype was characterised by the plants becoming infected and then recovering. Young symptomless leaves remained virus free and resistant to subsequent infections by PMMV-S. Similar phenotypes have been observed in transgenic *N. tabacum* plants expressing either translatable or non-translatable coat protein ORFs from tobacco etch virus (TEV) (Lindobo *et al.*, 1993; Dougherty *et al.*, 1994). It was suggested that inducible RNA-specific degradation was responsible for the resistance.

Two further examples of protection require consideration. Expression of the TMV 30 kDa cell-to-cell movement protein in transgenic plants does not offer protection against the virus (Deom *et al.*, 1987). However Lapidot *et al.* (1993) reported that transgenic *N. tabacum* plants expressing a dysfunctional TMV-U1 30 kDa protein were resistant to some *Tobamovirus* particles and TMV-U1 RNA. The ability of movement proteins to complement movement deficient mutants from different groups (Section 1.7.2) suggested that the resistance may be widespread. The resistant nature of transgenic plants expressing either functional or dysfunctional cell-to-cell movement proteins was tested using a range of viruses (Cooper *et al.*, 1995). These including a *Tobravirus*, *Caulimovirus*, *Nepovirus Bromovirus* and *Cucumovirus*. Following the inoculation of transgenic plants expressing a dysfunctional movement protein, little resistance was observed in leaves inoculated with viruses other than TMV. However, systemically infected leaves demonstrated varying degrees of resistance characterised by reduced symptoms, virus accumulation and spread. In contrast, infection was accelerated in plants which expressed a functional protein. Finally, antisense constructs complementary to the TMV-U1 5' untranslated leader sequence (Section 1.6.3) have been designed and transformed into the genome of *N. tabacum* plants (Nelson *et al.*, 1993). The acquired resistance following inoculation

with TMV particles resulted in a significant reduction in both symptom formation and virus accumulation. One benefit of the antisense method is that small, yet effective, constructs can be designed. As a result, the risk of viruses overcoming the resistance or even exploiting the constructs may be reduced (Nelson *et al.*, 1993).

1.10 OUTLINE OF RESEARCH

A primary aim of the research project was to investigate resistance to TMV. Initially interest was centred on the *Tm-1* gene. A long term goal was to clone the gene using insertional mutagenesis. It was hoped that this may be achieved, in collaboration with other laboratories, using an *Ac* or *Ac/Ds* (Activator/Dissociation) system (reviewed by Haring *et al.*, 1991). In order to facilitate a transposon tagging strategy, a simple yet reliable method was required for identifying whether the *Tm-1* gene was able to function. The loss of function in plants expressing the gene would indicate that a transposable element had inserted within the desired ORF. Evidence suggests that, the *Tm-1* gene interacts with the TMV replicase proteins to inhibit viral replication and the generation of subgenomic RNAs (section 1.9.2). The development of a TMV based vector capable of demonstrating replication-dependent expression of a marker gene was therefore seen as an attractive objective. Having received a 35S promoter driven cDNA clone of TMV-U1, it was essential to establish its ability to infect plants. The results of such investigations have been presented in Chapter 3. The generation and analysis of a number of replication-marker constructs which could have been applied to a transposon tagging initiative has been described in Chapters 4 and 5. It was intended to transform suitable constructs into the genomes of plants. If expressed in every cell of a plant genes which aided movement, and as a result infection, could be removed or inactivated. It was hoped that this would reduce the risk of TMV-based constructs escaping into the field. In addition to their application in a transposon tagging initiative, it was hoped that replication-marker constructs would have additional uses. They could be applied to many studies where replication and gene expression. needed to be observed and/or quantified. In addition to generating the replication-marker constructs, a number of constructs suitable for studying resistance to TMV have been generated. In particular, the application of TMV-based replication and replicase constructs to studies of *Tm-1* and *N* gene-mediated resistance has been described (Chapters 6 and 7, respectively). As a result, a number of interesting transgenic plants have been generated. It is likely that further studies of these plants may provide valuable information concerning *N* gene mediated resistance. In addition, studies may contribute to understanding resistance in transgenic plants, conferred by sequences from the TMV genome.

CHAPTER 2

MATERIALS AND METHODS

2.1 SOURCE OF CHEMICALS AND REAGENTS

The source of many of the chemicals and reagents, including enzymes and antibodies, have been referred to in the appropriate sections of text. Unless otherwise stated general chemicals and reagents were an analytical grade and purchased from Sigma Chemical Company, BDH Chemicals, and Fisher Scientific (formally Fisions Scientific Chemicals). Chemicals for the growth of bacterial cultures were purchased from Oxoid (Unipath) and Difco Laboratories. Chemicals and hormones for plant tissue culture were purchased from either Sigma Chemical Company, Flow Laboratories or Imperial Laboratories. α ^{32}P dCTP, and α ^{35}S dATP were purchased from Amersham International. Unless otherwise stated, all items were purchased from suppliers within the UK.

2.2 PLANT MATERIAL

All plants were grown either under greenhouse conditions or in a plant growth room, with a maximum daylength of 16 hours and daytime temperature of 25°C. Experiments were carried out using the plant lines described below.

Tobacco: *Nicotiana benthamiana* (*N. benthamiana*), *Nicotiana tabacum* cv. Petite Havana SR1 (*N. tabacum* SR1), and *Nicotiana tabacum* cv. Samsun *NN* (*N. tabacum* Samsun *NN* possessing the dominant *N* gene conferring resistance to TMV.

Tomato: *Lycopersicon esculentum* cv. Mill Craigella GCR 26 (+/+) (*L. esculentum* GCR 26 (+/+)), and near isogenic Lycopersicon *esculentum* cv. Mill Craigella GCR 237 (*Tm-1/Tm-1*) (*L. esculentum* GCR 237 (*Tm-1/Tm-1*)) which is homozygous for the *Tm-1* gene conferring resistance to TMV.

Pea: *Pisum sativum* (*P. sativum*)

2.3 PLASMIDS

Constructs were built using the following commercially available plasmids:

pBluescript SK$^-$(pBS SK$^-$) supplied by Stratagene
pcDNA II supplied by Invitrogen, The Netherlands
pSL301 supplied by Invitrogen, The Netherlands
pGEM T supplied by Promega
pBin 19 supplied by Clontech Laboratories

2.4 GROWTH AND MAINTENANCE OF PLASMIDS IN *ESCHERICHIA COLI*

2.4.1 Strains and Genotypes.

Three strains of *Escherichia coli* (*E. coli*) were used for the propagation of plasmids.

XL1-Blue: *recA1 endA*1 *gyrA*46, *thi hsdR*17 *supE*44 *relA*1 *lac*$^-$ *F'[ProAB*$^+$ *LacI*^{q} *lacZ*ΔM15 Tn*10*(*tet*^{r})]
JM109: *endA*1 *recA*1 *gyrA*96 *thi hsdR*17 *relA*1 *supE*44 *Δ(lac-proAB) F'[taD*36 *proAB*$^+$ *lacI*^{q} *lacZ*ΔM15]
HB101: *supE*44 *hsdS*20($r_B^-m_B^-$) *recA*13 *ara*-14 *proA*2 *lacY*1 *galK*2 *rpsL*20 *xyl*-5*mtl*-1

2.4.2 Antibiotic Aided Selection

E. coli cells harbouring the desired plasmids were selected with the aid of antibiotic resistance genes. 1000x stock solutions were normally prepared and stored at $^-$20°C. Antibiotics soluble in distilled water (dH_2O) were filter sterilised, using disposable 45 μm filters, prior to use.

Antibiotic	Solvent	Working dilution
Ampicillin	dH_2O	100 μg ml^{-1}
Chloramphenicol	ethanol	25 μg ml^{-1}
Kanamycin	dH_2O	100 μg ml^{-1}
Tetracycline	methanol	12.5 μg ml^{-1}

2.4.3 Preparation of Solid and Liquid Media

Media was made according to the following recipes. Solid media also contained 1.5 % agar which was added prior to autoclaving. Autoclaving was carried out for 20 minutes at 121°C, 15 lb per square inch.

Nutrient broth (NB) and Nutrient agar (NA) Prepared using either NB or NA powder according to the manufacturer's instructions. Media was sterilised by autoclaving prior to use.

Luria-Bertani Medium (LB) One litre of medium required the following ingredients:

Tryptone	10 g
Yeast Extract	5 g
NaCl	10 g

pH of the medium was adjusted to 7.5 using 5M NaOH, it was then bottled and sterilised by autoclaving.

Terrific Broth (Tartof and Hobbs, 1987). A 900 ml solution was prepared using the following ingredients:

Tryptone	12.0 g
Yeast Extract	24.0 g
Glycerol	4.0 ml

The medium was sterilised by autoclaving. Prior to use a sterile 100 ml solution containing 2.31 g (0·17 M) KH_2PO_4 and 12.54 g (0·72 M) K_2HPO_4 was added per litre of medium.

Minimal Medium M9 salts were prepared using the following ingredients per litre:

$Na_2HPO_4 \cdot 7H_2O$	12.8 g
KH_2PO_4	3.0 g
NaCl	0.5 g
NH_4Cl	1.0 g

If solid medium was required agar was added prior to autoclaving. The following sterile solutions were then added per litre of M9 salts.

20 % glucose	10.0 ml
1M $MgSO_4$	2.0 ml
1M $CaCl_2$	0.1 ml
1M thiamine-HCl	1.0 ml

2.4.4 Growth of Colonies on Solid Media

Following sterilisation, by autoclaving, solid media was allowed to cool to ~50°C before adding the appropriate antibiotics. Working in a lamina flow cabinet, the media was then poured into sterile Petri dishes and left to solidify. Single colonies

were grown by dipping a flame sterilised loop into the appropriate culture (glycerol stock, colony or liquid culture) and then streaking it on to an agar plate. The plate was inverted and incubated overnight at 37°C. Alternatively cells suspended in a small amount of liquid broth were spread over a plate using a flame sterilised glass spreader, once dry inverted plates were incubated overnight at 37°C.

2.4.5 Growth of Liquid Cultures

Working in a laminar flow cabinet, 5 ml-25 ml of liquid media was transferred to a sterile universal or conical flask and the appropriate antibiotics were added. A sterile pipette tip was then used to inoculate the media by selecting a single colony from an agar plate (occasionally media was inoculated directly from a glycerol stock). Cultures were grown overnight or to the required optical density in an orbital shaker (200 revolutions per minute) at 37°C. Where necessary these small cultures were used to inoculate lager volumes of media (100-500 ml).

2.4.6 Growth of Cultures in the Presence of Chloramphenicol

It was sometimes desirable to inhibit the further replication of bacterial cells, while continuing to amplify plasmid DNA, using the drug chloramphenicol. Following the protocol described in Sambrook *et al.* (1989), 500 ml of Terrific broth was transferred to a 2 litre conical flask and the appropriate antibiotics were added. The media was inoculated using a 25 ml overnight culture and incubated in an orbital shaker at 37°C for 21/2 hours. At this point it was assumed that the absorbance at 600 nm (A_{600}) was ~0·4. The culture was then inoculated using 2·5 ml from a stock solution (34 mg ml^{-1}) of chloroamphenicol. The flask was returned to the orbital shaker and incubated overnight at 37°C.

2.4.7 Storage of Bacteria

It was possible to store colonies on inverted agar plates, at 4°C, for up to one month. For long term storage glycerol stocks were made. 250 µl of sterile glycerol was added to 750 µl of fresh overnight culture in a cryotube. The tube contents was flash frozen in liquid nitrogen and stored at -80°C. In addition to glycerol stocks purified plasmid stocks of all constructs were stored at -20°C.

2.4.8 Preparation of Competent Cells

The method used for the preparation of competent cells able to take up foreign

DNA was based on that of Cohen *et al.* (1972). Using this method aliquots of cells are capable of producing ~10^7 transformed colonies per μg of supercoiled DNA (Sambrook *et al.*, 1989).

The desired strain of *E. coli* was grown on a M9 minimal medium plate and a single colony was chosen to inoculate 5 ml of LB. The cells were grown overnight and then used to inoculate 100 ml of sterile LB. The flask was incubated in an orbital shaker at 37°C until the A_{600} was 0·6. Care was taken to keep the cells at 4°C for the remainder of the procedure. The culture was transferred to sterile 50 ml tubes and centrifuged at 2 000 x*g* for 10 minutes in a benchtop centrifuge. The medium was discarded and the pellets gently resuspended in 1/2 the original volume of sterile ice cold $CaCl_2$ (50 mM). The cells were then incubated on ice for one hour prior to centrifugation at 2 000 x*g* for 10 minutes. The pelleted cells were finally resuspended in 1/20 original volume of sterile ice cold $CaCl_2$ (50 mM). For short term storage, up to one week, the cells were kept at 4°C. Evidence suggests that storage at 4°C in $CaCl_2$ for 48 hours can improve competence (Dagert and Ehrlich, 1979). Long term storage required glycerol to be added to a final concentration of 20 %, 200 μl aliquots were then flash frozen in liquid nitrogen and stored at -80°C.

2.4.9 Transformation of Competent Cells

Ensuring that cells remained on ice, 1 μg of purified plasmid, or 10 μl from a ligation reaction (see Section 2.5.10), was added to a 200 μl aliquot of competent cells in a microcentrifuge tube. Following incubation on ice for 30 minutes the cells were heat shocked. This was carried out by transferring the tube to 42°C for 90 seconds and then directly back to ice for a further 5 minutes. 1 ml of LB was added to the tube which was then incubated in an orbital shaker at 37°C for 1 hour. The cells were pelleted by microcentrifugation at 12 000 x*g* for 5 minutes and resuspended in 200 μl of fresh LB. A flame sterilised glass spreader was used to distribute the cells over a fresh LA plate containing appropriate antibiotics. Once dry inverted plates were incubated overnight at 37°C.

2.5 MANIPULATION OF PLASMID DNA

2.5.1 Purification of Plasmid DNA

The method followed to lyse bacterial cells was based on alkali-lysis protocols described by Birnboim and Doly (1979) and Ish-Horowicz and Burke, (1981). Methods for purification are based on those described by Sambrook *et al*, (1989).

2.5.1.1 Solutions Required for Alkaline-Lysis

Lysis Buffer:

Tris.Cl pH 8.0	25 mM
EDTA	10 mM
Sucrose	500 mM

Tris[hydroxymethyl]aminomethane (Tris), Ethylenediaminetetraacetic acid (EDTA)

Alkaline-SDS:

NaCl	200 mM
Sodium dodecyl sulphate (SDS)	1 % (w/v)

Potassium acetate: 3M potassium and 5M acetate

2.5.1.2 Small Scale Preparation of Crude Plasmid DNA

Using an overnight culture, 1.5 ml was transferred to a microcentrifuge tube and the cells were collected by centrifugation at 12 000 x*g* for 5 minutes. The supernatant was removed and the pellet resuspended in 100 µl of lysis buffer. 200 µl of alkaline-SDS was added to the tube and the contents were mixed by inversion. 150 µl of potassium acetate was then added and the contents again mixed by inversion. Chromosomal DNA and protein was pelleted by microcentrifugation at 12 000 x*g* for 10 minutes. The supernatant was removed to a fresh tube and mixed with an equal volume of propan-2-ol. Plasmid was collected in a pellet by microcentrifugation at 12 000 x*g* for 10 minutes. The pellet was resuspended in 400 µl of dH_2O containing 1 µl RNase A (from a 10 mg ml^{-1} stock which had been boiled for 10 minutes to destroy DNase enzymes).

2.5.1.3 Phenol:Chloroform Extractions

Phenol:chloroform extractions were performed in order to clean the plasmid DNA, resuspended in dH_2O. 1/2 volume phenol and 1/2 volume chloroform was added to the DNA and mixed by mechanical vortex. The top (aqueous) phase containing the plasmid was separated by microcentrifugation at 12 000 x*g* for 5 minutes and removed to a fresh tube. Phenol:chloroform extractions were repeated until the aqueous phase remained clear.

2.5.1.4 Ethanol Precipitation

DNA was precipitated by the addition of salts (either 1/10 volume 3 M sodium acetate, pH 5.2 or 1/4 volume 10 M ammonium acetate) and 21/2 volumes of ice cold ethanol. Following incubation at -80°C for 10 minutes plasmid was collected by microcentrifugation at 12 000 x*g* for 10 minutes. The DNA pellet was washed with 200 µl of ice cold 70 % ethanol and microcentrifuged for a further 2 minutes. Finally the pellet was dried in a vacuum desiccator and resuspended in an appropriate volume (10-50 µl) of dH_2O water.

2.5.1.5 Large Scale Preparation of High Quality Plasmid DNA

PEG:NaCl Solution:

NaCl	1.3 M
Polyethylene glycol (PEG) (Average M_r = 8000)	13 % (w/v)

Cells from a 100-500 ml overnight culture were collected by centrifugation at 2 500 x*g* for 20 minutes using suitable centrifuge tubes. The pellet was resuspended in 2-10 ml (known as the original volume) of lysis buffer. 2x the original volume of alkaline-SDS was added and mixed by inversion. The solution was placed on ice for 5 minute, lysis was complete when the solution cleared. 11/2x the original volume of potassium acetate was added, mixed by inversion and the solution placed on ice for a further 5 minutes. Protein and chromosomal DNA was pelleted by centrifugation at 2 500 x*g* for 15 minutes. The supernatant was removed to a fresh tube and plasmid precipitated by the addition of 0.6 volumes of propan-2-ol. The tube contents were mixed and incubated at room temperature for 10 minutes. Following centrifugation at 2 500 x*g* for 15 minutes the pellet was resuspended using 0.5 ml dH_2O per 100 ml of original culture. The addition of an equal volume of ice cold 6M lithium chloride was used to precipitate high molecular weight RNA. This was collected by centrifugation at 2 500 x*g* for 15 minutes. Plasmid DNA was precipitated by mixing it with an equal volume of propan-2-ol followed by incubation at room temperature for 10 minutes. The DNA was collected by centrifugation at 2 500 x*g* for 15 minutes. The resulting pellet was washed in 70 % ice cold ethanol, dried in a vacuum desiccater and resuspended in 100 µl dH_2O containing 5 µl RNase A (from a 10 mg ml^{-1} stock). RNA was digested during a 30 minute incubation at 37°C. The plasmid DNA was then precipitated by adding 100 µl of PEG:NaCl solution and incubating on ice for 10 minutes. Following microcentrifugation at 12 000 x*g* for 10 minutes the pellet was resuspended in 100 µl

dH_2O. DNA was extracted using phenol:chloroform and precipitated using ethanol and ammonium acetate. The pellet was finally resuspended in an appropriate volume of distilled water (50-250 µl equalled ~1 µg μl^{-1}).

2.5.2 Calculating the Concentration of Plasmid DNA

The concentration of plasmid DNA was estimated by resolving fragments of digested plasmid (Section 2.5.3) on an agarose gel (Section 2.5.4). DNA markers of a known concentration were resolved at the same time. The intensity of bands was then compared.

Alternatively the concentration was determined spectrophotometrically by measuring the wavelength of UV irradiation absorbed. Purified plasmid was diluted (1:250-1:1000) using dH_2O and transferred to a quartz cuvette. It was then scanned with wavelengths ranging from 200 to 300 nm. The resulting absorbance profile, with a peak at 260 nm, gave an indication of plasmid purity. The A_{260} was used to calculate DNA concentration given that an $A_{260} = 1$ is equivalent to 50 µg ml^{-1} of dsDNA, 40 µg ml^{-1} of RNA or ssDNA or 20 ug ml^{-1} of single-stranded oligonucleotides (Sambrook *et al.*, 1989). It should be noted that contaminants including purification reagents and ribonucleic acids can undermine the accuracy of spectrophotometric readings.

2.5.3 Restriction Enzyme Digests

Restriction endonuclease enzymes were purchased from either Gibco BRL or New England Biolabs. Digests were set up in volumes of 15 µl or 25 µl. They contained 0·1-2.0 µg of plasmid DNA, a 10x excess of the required restriction enzymes, and an appropriate amount of the manufacturer's 10x buffer. Incubation was carried out for 2-4 hours at 37°C (or at the temperature recommended by the manufacturer). Digested DNA was then checked along side undigested DNA by agarose gel electrophoresis.

2.5.4 Agarose Gel Electrophoresis

10x Tris-acetate EDTA (TAE):

Tris.acetate, pH 8.0	400 mM
EDTA	10 mM

5x sample loading buffer:

TAE	5x
Glycerol	10% (v/v)
Bromophenol blue	0.01% (w/v)

A 0.8-1.0 % (w/v) agarose gel was prepared by heating agarose in 1x TAE. It was allowed to cool, to below 50°C, before adding the fluorescent dye ethidium bromide (0.5 μg ml^{-1}) (Sharp *et al.*, 1973). The gel was then poured into a sealed casting tray containing a suitable comb to form the wells. Once set it was transferred to an electrophoresis tank containing 1x TAE. Samples were mixed with 1/4 their volume of 5x loading buffer and pipetted into individual wells. At the same time DNA molecular weight markers (1 KB ladder, purchased from Gibco BRL) were pipetted into a separate well to indicate the size of DNA fragments. Electrophoresis was usually carried out at 100 v and DNA was visualised under UV light.

2.5.5 Purification of DNA Fragments Excised From Agarose Gels

2.5.5.1 Using a Gene Clean Kit (Bio 101 Ltd., USA).

Fragments between 200 bp and 10 kb were purified using a Gene Clean kit, according to the manufacturer's protocol. Gel slices were melted in 250-500 μl of sodium iodide by incubating at 55°C for 5 minutes. 5-10 μl of glass milk was then added and mixed by vortex. Incubation on ice for 10 minutes allowed the DNA to bind to the glass beads. The tube was then centrifuged at 12 000 x*g* for 30 seconds and the supernatant discarded. The DNA coated glass beads were resuspended by vortex in 750 μl of ice cold New Wash and collected by microcentrifugation at 12 000 x*g* for 30 seconds, the supernatant was discarded. The New Wash step was repeated three times, in total. Excess fluid was then carefully removed from the pellet using a pipette. The pellet was resuspended in 25 μl dH_2O and heated at 55°C for 2 minutes, this released the DNA from the glass beads. The tube was microcentrifuged at 12 000 x*g* for 5 minutes and the supernatant, containing the DNA, removed to a fresh tube. A small amount of the purified DNA could be visualised by agarose gel electrophoresis.

2.5.5.2 By Electroelution

High salt binding solution:

Sodium acetate	3 M
Bromophenol blue	0.01 % (w/v)

DNA fragments >10 kb were purified by electroelution, using a specially designed electroelution tank. The tank was filled with 1x TAE and the isolated gel slice was placed into a well. A current was passed through the tank and carried DNA from the agarose gel slice into a neighbouring well containing 200 µl of high salt binding solution. Following electroelution at 100 v for a least 2 hours the DNA in the high salt binding solution was precipitated using 2.5 volumes of ice cold ethanol. The pellet was resuspended in 10-25 µl dH_2O.

2.5.6 Dephosphorylation of Vector DNA

If plasmid vectors were prepared for cloning using only one restriction enzyme, it was necessary to remove the phosphate group from the 5' end of the linear DNA. This reduced the frequency of the compatible ends rejoining without an insert in the presence of DNA ligase. The volume of a complete restriction enzyme digest was made up to 50 µl using dH_2O. 0·5 µl (1 unit) of calf intestinal alkaline phosphatase (purchased from Promega) was then added to the digest. Dephosphorylation was allowed to proceed at 37°C for 10 minutes. The enzyme was then inactivated by the addition of 2 µl EDTA (0·5 M, pH 8). The dephosphorylated vector was cleaned by phenol:chloroform extraction and precipitated using ethanol.

2.5.7 Filling in 5' Protruding Ends

Following restriction enzyme digestion, 5' protruding ends were filled in using the Klenow fragment of DNA polymerase I (purchased from Gibco BRL). Digested DNA was purified and resuspended in 23 µl of dH_2O. The following components were the added - 1 µl from a 10 mM stock of deoxyribonucleosides (dNTPs) (prepared by mixing equal ratios of dATP, dCTP, dGTP and dTTP) and 1 µl (0·1 units) of Klenow. The reaction was incubated at 37°C for 30 minutes and then at 70°C for 5 minutes to inactivate the enzyme. The blunt-ended DNA was cleaned by phenol:chloroform extraction and precipitated using ethanol.

2.5.8 Blunting 3' Protruding Ends

The bacteriophage enzyme T4 DNA polymerase has 5' to 3' polymerase activity and 3' to 5' exonuclease activity. At 12°C these two activities are approximately equal. In order to remove the 3' protruding ends T4 DNA polymerase was used in conjunction with dNTPs to ensure that the ends remained blunt. Digested DNA was purified and resuspended in 20 µl of dH_2O, the following components were then added - 1 µl from a 10 mM stock of dNTPs (prepared by mixing equal ratios of

dATP, dCTP, dGTP and dTTP) and 1 µl (5 units) of T4 DNA polymerase (purchased from Gibco BRL). The reaction was incubated at 12°C for 15 minutes, DNA was then purified by phenol:chloroform extraction and precipitated using ethanol.

2.5.9 Amplification of DNA Using the Polymerase Chain Reaction

Specific regions of DNA were amplified using the Polymerase chain reaction (PCR). This made use of thermo-stable *Taq* DNA polymerase I, isolated from *Thermus aquaticus* (Chien *et al.*, 1976), so that different heat regimes could be applied in a thermal cycler (Saiki *et al.*, 1988). Two primers were required to bind to sites on opposite strands of DNA. One site was positioned at either end of the fragment to be amplified. The procedure set out below is based on Innis *et al.* (1990), minor adjustments to the procedure were made to suit specific requirements.

11x PCR buffer:

Tris.Cl, pH 8.8	494.4 mM
$(NH_4)_2SO_4$	123.0 mM
$MgCl_2$	49.6 mM
2-mercaptoethanol	75.0 mM
EDTA, pH 8.0	50.0 µM
Each dNTP	11.1 mM
BSA	1.26 mg ml^{-1}

dNTPs were purchased from Promega. PCR reactions were prepared in 1x PCR buffer and were made up to a total volume of 20 µl when amplifying from plasmid (50 µl, when amplifying from bacterial colonies or plant genomic DNA).

PCR reaction:

Template DNA	10 ng
Each Primer	200 ng
Taq DNA Polymerase I	2 units

Taq DNA polymerase I was purchased from either Perkin Elmer or Promega. Primers were prepared by the Protein and Nucleic Acid Chemistry Laboratory, University of Leicester. Each reaction was overlaid with mineral oil to prevent evaporation.

Thermal cycles (using a Perkin Elmer Cetus DNA Thermal Cycler)

Step	Temperature	Duration
Denaturing	94°C	1 minute
Annealing primers	48-60°C depending on sequence	1 minute
Extension	72°C	1 minute for each kb

The cycle was repeated 25-30 times. Reactions were removed from beneath the mineral oil. A small amount was then visualised using agarose gel electrophoresis and the remainder was cleaned by phenol:chloroform extraction.

2.5.10 Ligations

T4 DNA ligase (purchased from Gibco BRL) was used to ligate compatible or blunt ended DNA fragments. Each ligation reaction was in a volume of 25 µl and contained the following components - 0.1-0.5 µg of vector DNA , an approximately equimolar amount of insert DNA (the exact ratio depended on the size of both the vector and the insert), 1 µl (5 Weiss units) of bacteriophage T4 DNA ligase and 5 µl of the manufacturer's 5x ligation buffer. A control ligation was set up simultaneously, containing vector alone. The control was used to judge the frequency of the vector rejoining without the required insert. Ligations were incubated at room temperature for 2-4 hours, prior to transformation into competent cells (Section 2.4.9).

2.5.11 Identification of a Correctly Cloned Product

A number of different methods were used to identify cells that had been successfully transformed with the correct plasmid vector and insert.

2.5.11.1 Blue/White Colour Selection

If cloning into a M13 plasmid vector for the first time, the β-Galactosidase (lac Z) gene was used for blue/white colour screening. Expression of the lac Z gene in the presence of substrates 5-Bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) and Isopropylthio-β-D-galactoside (IPTG) resulted in the growth of blue cells. Where the gene had been interrupted by a cloned insert the cells were white. Transformed cells

were spread on plates containing 50 µl X-Gal (from 20 mg ml^{-1} stock in dimethylformamide) and 20 µl IPTG (from a 200 mg ml^{-1} stock).

2.5.11.2 Colony Screening by Hybridisation

This method was particularly useful if colour selection was not possible. Two agar plates, containing the correct antibiotics for plasmid selection, were prepared. One was overlaid with nylon membrane (Hybond N, Amersham International) on which a numbered grid had been marked. The second plate (known as the reference plate) was marked with a similar grid. Using sterile pipette tips colonies of interest were streaked on to a square on the membrane grid and then on to the corresponding square of the reference plate. Both plates were incubated at 37°C overnight. Following the growth of colonies, the membrane was placed, colony side down on, 3MM paper (Whatman International) soaked in cell lysis solution (2x SSC (see Section 2.5.12), 0.5 % (w/v) SDS) for 30 seconds. Once dry, DNA was fixed to the membrane using a programmed UV cross linking machine (Stratagene UV Stratalinker 2400). Hybridisation of a radioactive probe to the desired insert was carried out according to the methods described in Section 2.7. Colonies containing this insert were then identified and selected from the reference plate.

2.5.11.3 PCR Direct from Colonies

This method was suitable for preliminary diagnosis, as long as the desired insert contained suitable PCR primer sites. PCR reactions were set up as described in Section 2.5.9. However, instead of adding purified template DNA a sterile pipette tip was used to select the colony of interest. This tip was used to infect the PCR reaction, and then to transfer the cells on to a reference agar plate. The inverted plate was incubated overnight at 37°C. PCR cycles were as described in Section 2.5.9. Following agarose gel electrophoresis, colonies from which the expected product had been amplified were selected from the reference plate.

2.5.11.4 Plasmid Preparation and Restriction Enzyme Digests

Final diagnosis was always carried out by preparing plasmid from an overnight culture and performing suitable, diagnostic restriction enzyme digests.

2.6 SOUTHERN BLOTTING

It was sometimes necessary to confirm the identity of DNA fragments by transferring them from an agarose gel to Hybond N membrane and then probing them with a suitable fragment of radiolabelled DNA (see Section 2.7). DNA was transferred by capillary action using a method based on that of Southern (1975).

Depurinating solution:

HCl	0.2 M

Denaturing solution:

NaOH	0.5 M
NaCl	1.5 M

Neutralising solution:

Tris.Cl, pH 7.4	1.0 M
NaCl	1.5 M

20x sodium chloride
sodium citrate (SSC):

NaCl	3.0 M
$Na_3C_6H_5O_7.2H_2O$	0.3 M

Following agarose gel electrophoresis, the gel was visualised under UV light and photographed with a ruler to indicate the position of individual DNA fragments. Unwanted areas were removed and the gel was placed in a suitable container to allow agitation on a rotary shaker. If the transfer of DNA fragments >10 kb was required the gel was soaked in depurinating solution for 10 minutes (with gentle agitation), this step was omitted for smaller fragments. The gel was then soaked in denaturing solution for 30 minutes, followed by neutralising solution for a further 30 minutes. Hybond N membrane, the same size as the gel, was prepared by soaking it first with dH_2O and then with 2x SSC. Using 3MM paper, five pieces were cut to the size of the gel, one was soaked in 2x SSC. A sponge covered in 3MM paper was placed in a tray containing 20x SSC, the gel was placed on top of the sponge and surrounded with cling film. The membrane was then placed over the gel taking care to exclude air bubbles. The membrane was covered in the 2x SSC soaked piece of 3MM paper followed by the four dry pieces and a pile of absorbent paper towels (approximately 4 cm high). Finally an ~500 g weight was placed on top of the 3MM paper. The blot was left overnight to allow the transfer of DNA from the gel to the membrane. The

membrane then was allowed to dry, the DNA was fixed to the membrane by exposure to UV light in a crosslinking machine.

2.7 HYBRIDISATION OF RADIOLABELLED PROBES TO DNA OR RNA

2.7.1 Preparation of a DNA Probe

Radiolabelling was carried out using random primers to initiate DNA polymerisation along single-stranded template DNA. The method is based on that of Feinberg and Vogelstein (1984).

2.7.1.1 Preparation of Oligo-labelling Buffer (OLB)

Buffer A:

2 M Tris.Cl, pH 8.0	625 µl
5 M $MgCl_2$	25 µl
2-mercaptoethanol	18 µl
0.1M dATP	5µl
0.1 M dGTP	5 µl
0.1 M dTTP	5 µl
dH_2O	350 µl

Buffer B: 2M N-[2-hydroxyethyl]piperazine-N'-[2-ethane-sulphonic acid] (HEPES), adjusted to pH 6.6 using NaOH

Buffer C: Randomhexadeoxriboynucleotides, A_{260}=90 units ml^{-1} suspended in;

Tris.Cl, pH 7.0	3.0 mM
EDTA, pH 7.0	0.2 mM

dNTPs and oligonucleotides were purchased from Pharmacia Biotech. 5x OLB was prepared by mixing the buffers A, B, and C using the ratio 2:5:3.

2.7.1.2 The Oligo-Labelling Reaction

Digested DNA fragments were purified and used to prepare radiolabelled probes. Alternatively PCR amplified DNA fragments could have been used. ~10 ng

of purified DNA was suspended in 9.8 µl of dH_2O in a screw capped microcentrifuge tube. The DNA was denatured, immediately prior to setting up the reaction by boiling for 5 minutes and then cooling on ice for 5 minutes. A brief microcentrifugation ensured that the DNA was collected at the bottom of the tube. The following reagents were then added:

5x OLB	3 µl
10 mg ml^{-1} BSA (DNase free)	0.6 µl
α 32P dCTP (0.37 MBq $µl^{-1}$)	1 µl
Klenow fragment of DNA polymerase I	0.6 µl (1 unit)

The Klenow fragment of DNA polymerase I was purchased from Pharmacia Biotech. The reaction was incubated at 37°C for 2 hours.

2.7.1.3 Measuring the Incorporation of Radiolabel

Following incubation for 2 hours, 85 µl of dH_20 was added to the oligo-labelling reaction. 1 µl of the diluted reaction was pipetted on to a glass microfibre disc (GF/C disc, Whatman International Ltd.), this was used to determine the total amount of radiolabel. A second 1 µl was removed from the diluted reaction and added to 500 µl of herring sperm DNA (500 µg ml^{-1} dissolved in 20 mM EDTA) and 125 µl of 50 % (w/v) trichloroacetic acid (TCA). The DNA was allowed to precipitate on ice for 5 minutes, it was then collected, by vacuum filtration, on to a glass microfibre disc. The disc, used to determine incorporated radiolabel, was washed twice with 5 ml of 10 % TCA (w/v) and then 5 ml of industrial methylated spirits. The two discs were placed in separate vials containing 2 ml of liquid scintillant (Opti Fluro O, Packard Instrument Company Inc.). The vials were passed through a LKB Wallac liquid scintillation counter, percentage incorporation was calculated from the readings obtained.

2.7.2 Hybridisation

100x Denhardts Reagent:

BSA	2 % (w/v)
Ficoll 400	2 % (w/v)
polyvinylpyrrolidone (PVP)	2 % (w/v)

Herring sperm DNA: A 10 mg ml^{-1} stock was prepared and sheared, by passing it 10 times through a hypodermic needle. It was boiled for 5 minutes, immediately prior to use and then placed on ice for 5 minutes.

DNA prehybridisation solution:

SSC (Section 2.6)	6x
Denhardts Reagent	5x
SDS	0.5% (w/v)
Herring sperm DNA	100 μg ml^{-1}

Prehybridisation and hybridisation were carried out at 65°C.

RNA prehybridisation solution:

SSC	6x
Denhardts Reagent	5x
Formamide	50 % (v/v)
NaH_2PO_4	50 mM
Herring sperm DNA	100 μg ml^{-1}

Prehybridisation and hybridisation were carried out at 42°C.

Hybond N membrane was blocked in order to reduce non-specific binding, by incubating in prehybridisation solution for 2 hours. The temperature and choice of solution depended on whether the probe was being hybridised to DNA or RNA. The radiolabelled probe was denatured by boiling for 5 minutes and then cooling on ice for a further 5 minutes. It was then added to the prehybridisation solution. The probe was left to hybridise overnight with gentle agitation.

2.7.3 Washing the Filters and Exposure to X-ray Film

Wash A (high stringency):

SSC	3x
SDS	0.1 % (w/v)

Wash B (low stringency):

SSC	0.5x
SDS	0.1 % (w/v)

Typically, filters were washed twice for 15 minutes in wash A, followed by twice for 15 minutes in wash B. However, they were regularly monitored using a Geiger counter and the stringency of washes was adjusted accordingly. Once washed, the moist filters were wrapped in Saran Wrap (Dow Chemical Company, USA), and exposed to X-Ray film (either Hyperfilm-MP from Amersham International, or X-ray film from Genetic Research Instrumentation Ltd.). During exposure to film the filters were stored in an X-Ray cassette at -80°C. Film was developed using standard X-Ray developing and fixing solutions (Champion Photochemicals). If necessary probe was removed from Hybond N membrane following the manufactures protocol.

2.8 DNA SEQUENCING

Sequencing was based on the method described by Sanger *et al.* (1977). DNA polymerase was used for primer extension reactions, these underwent base specific termination by the incorporation of dideoxyribonucleotides. High quality double-stranded template DNA was prepared according to the method described in Section 2.5.1.5. It was essential to remove all traces of PEG. Therefore, following the precipitation of DNA using PEG solution the pellet was washed using 70 % ice cold ethanol.

2.8.1 Manual Sequencing

2.8.1.1 Preparing the Reactions

Sequencing reactions were prepared using a ^{T7}Sequencing kit (Pharmacia Biotech), according to the manufacturer's protocol. This involved denaturing ~2 µg of double-stranded template DNA using 2 M NaOH. ~25 ng of an appropriate oligonucleotide primer was annealed to the template. Labelling reactions were carried out using T7 DNA Polymerase I and incorporating α^{35}S dATP. Each reaction was terminated by the random incorporation of an appropriate dideoxyribonucleotide and finally by the addition of stop solution. The stop solution contained bromophenol blue so that progress could be monitored during electrophoresis.

2.8.1.2 Sequencing Gel Preparation and Electrophoresis

10x Tris-borate EDTA (TBE):

Tris.borate, pH 8.3	900 mM
EDTA	20 mM

Sequencing gel mix:

Acrylamide	34.11 g
Bis-acrylamide	1.8 g
Urea (ultra pure)	252 g

N,N'-methylene-bis-acrylamide (bis-acrylamide). The volume was adjusted to 540 ml, 60 ml 10x TBE was then added. The mixture was deionised using amberlite MB-1 monobase resin. Having been mixed with the solution for 30 minutes the resin was removed by filtration.

Using Sequi-Gen sequencing equipment (Bio-Rad Laboratories), the plates were cleaned and assembled according to the manufacture's instructions. Polymerised sequencing gel mix was used to seal the bottom of the plates. 480 µl of 10 % ammonium sulphate and 64 µl N,N,N',N'-tetramethylethylenediamine (TEMED) was added to 20 ml of sequencing gel mix. The mixture was then quickly poured into the casting tray containing the assembled plates. In order to prepare the sequencing gel 480 µl of 10 % ammonium sulphate and 64 µl of TEMED were added to 80 ml of sequencing gel mix. The mixture was carefully pipetted between the plates taking care to avoid air bubbles. An inverted sharks tooth comb was inserted at the top of the gel to form space for the wells, this was inserted in the correct manner once the gel had set. The polymerised gel was then assembled for electrophoresis and the buffer tanks filled with 1x TBE. Prior to loading samples the gel was preheated to 60°C and the wells were rinsed using TBE. Samples were boiled for 2 minutes, 3 µl of each sample was then immediately loaded into an appropriate well. Electrophoresis was carried out at ~2000 v, the blue dye front being used to measure progress. Once electrophoresis was complete the apparatus was disassembled and the plates gently separated. The gel was carefully absorbed on to a sheet of 3MM paper and dried using a vacuum gel drier. It was then exposed to X-ray film (Hyperfilm-MP Amersham) for at least 24 hours. X-ray cassettes were stored at room temperature and film was developed using X-ray developing and fixing solutions.

2.8.2 Automatic sequencing.

Sequencing was carried out using a *Taq* Dye Deoxy Terminator Cycle Sequencing Kit (Applied Biosystems) according to the manufacturer's protocol. The thermostable enzyme *Taq* DNA polymerase was used and reactions were labelled by the incorporation of a dye along side the terminating dideoxyribonucleotides. Reactions were carried out in a Perkin Elmer Cetus DNA Thermo cycler and analysed using the Applied Biosystems Model 373A DNA Sequencing System. Analysis was carried out

by the Protein and Nucleic Acid Chemistry Laboratory, University of Leicester.

2.8.3 Analysis of Data

Sequence data was analysed with the aid of two computer packages, SeqEd (Applied Biosystems) and Gene Jockey (Biosoft), provided by the University Molecular Biology Users Group.

2.9 *IN VITRO* ANALYSIS

2.9.1 *In Vitro* Transcription

In vitro transcription was carried out using an *in vitro* transcription kit purchased from Stratagene. This enabled the generation of mRNA from cDNAs which had been cloned into plasmids downstream of either the T3 or T7 promoter. Transcription was carried out according to the manufacturer's protocol. It involved incubating 1 µg of linearised plasmid at 37°C in the presence of ribonucleosides (rNTPs: ATP, CTP, GTP, UTP) and either T3 or T7 RNA polymerase. 7 µl from a 20 µl reaction was visualised on a agarose gel to check the success of the reaction. The remaining RNA was purified by phenol:chloroform extractions and precipitated using ethanol. The RNA pellet was resuspended in 20 µl diethyl pyrocarbonate (DEPC) treated dH_2O (to remove RNAses - 0.1 % (v/v) DEPC was added to the dH_2O, stirred for 30 minutes and then autoclaved).

2.9.2 *In Vitro* Translation

Following on from *in vitro* transcription, mRNA was translated into protein using wheat germ extract purchased from Promega. The extract is suitable for translation of viral, prokaryotic and eukaryotic mRNAs. All reagents were supplied by the manufacturer and reactions were carried out at room temperature according to the manufacturer's protocol. This involved incubating 1 µl of *in vitro* transcribed mRNA with wheat germ extract and an amino acid mixture. The product could be assayed by a number of procedures for example western immunoblot analysis or enzyme activity assays. Alternatively a radioactive label could be incorporated allowing visualisation on X-ray film following SDS-PAGE (Section 2.13.2.3). In this thesis *in vitro* translation was only used to produce the luciferase enzyme. As a result luciferase assays (Section 2.13.3) were performed on the products.

2.10 THE USE OF TMV

All TMV constructs generated and used as part of this thesis were based on the genome of TMV-U1. Where restriction enzyme sites have been described the number refers to the nucleotide immediately upstream from the point of cleavage. Where codons have been described the number refers to the first 5' nucleotide.

2.10.1 Manual Inoculation of Plants with TMV

The protocol was based on the optimised method reported by Takahashi (1956). Healthy young plants, 6-8 weeks old, were chosen for manual inoculation experiments. The upper surface of a 1/2-3/4 fully expanded leaf was dusted with carborundum (320 mesh silicon carbide powder). This acted as an abrasive and caused mechanical damage. The inoculum in a volume of 10-100 µl was gently rubbed on to the surface of the leaf. At 10 minutes post inoculation leaves were washed under running water to remove the carborundum. In order to contain plants infected with virus they were subsequently kept in Fisons growth cabinets.

2.10.2 Purification of TMV from Infected Plants

The method used, described by Gooding and Hebert (1967), predicted a harvest of 1g of TMV per 1 000 g of sytemically infected tissue.

TMV Extraction Buffer:

Na_2HPO_4-KH_2PO_4, pH 7.2	0.5 M
2-mercaptoethanol	0.1 % (v/v)

Storage Buffer:

Na_2HPO_4-KH_2PO_4, pH 7.2	10 mM

Infected plant material was harvested and frozen in liquid nitrogen. Using a blender, the frozen tissue was mixed with TMV extraction buffer (1 ml of buffer per gram of tissue) and homogenised for 5 minutes. The homogenate was then strained through muslin into a beaker. The solvent *n*-butanol (8 ml per 100 ml of extract) was added to the extract and stirred for 15 minutes to allow the chloroplasts to coagulate. The mixture was transferred to a centrifuge bottle and centrifuged at 10 000 x*g* for 30 minutes. The supernatant was removed to a fresh bottle and PEG (Average $M_r = 6\,000$) was dissolved in it at a ratio of 4 g per 100 ml of supernatant. Centrifugation was repeated at 10 000 x*g* for 15 minutes and the pellet was

resuspended in storage buffer (20 ml per 100 ml of initial extract). A second purification step was carried out by dissolving NaCl and PEG (average M_r = 6 000) in the virus suspension (4 g of each per 10 ml of suspension). Centrifugation, at 10 000 x*g*, was carried out for a further 15 minutes. The pellet was suspended in storage buffer (2 ml per 100 ml of initial extract), transferred to microcentrifuge tubes and clarified by microcentrifugation at 12 000 x*g* for 10 minutes. The supernatant containing purified TMV particles was removed to fresh tubes and stored at 4°C.

2.10.3 Isolation of TMV Coat Protein

Dissociation Buffer:

Na H_2PO_4, pH 7.0	0.02 M
SDS	2 % (w/v)
Dithiothreitol (DTT)	0.4 % (v/v)

A suspension of purified TMV particles was incubated with an equal volume of dissociation buffer at 90°C for 2 minutes. The dissociated coat protein was then stored at 4°C until required for SDS-PAGE (Section 2.13.2.3).

2.10.4 Identification of TMV Using Electron Microscopy

Samples were ground with liquid nitrogen in a microcentrifuge tube, dH_2O was then added (~1 ml g^{-1} tissue). Following centrifugation at 12 000 xg for 5 minutes the aqueous phase was removed to a fresh tube, leaving behind the plant debris. Transmission electron microscopy was carried out by staff in the Electron Microscopy Suite, University of Leicester.

2.11 TRANSIENT ASSAYS

2.11.1 Preparation and Inoculation of *N. tabacum* Protoplasts

The generation of protoplasts provides a method by which a population of simultaneously infected cells can be studied. Protoplasts (which lack a cell wall) do not occur naturally and must therefore be specially cultured in a hypertonic medium. Due to this the results obtained following their infection can be misleading (Takebe, 1975). However, if used in conjunction with other experimental techniques protoplasts provide a tool for the study of viral infections at the cellular level. The method used for the generation of *N. tabacum* leaf mesophyll cell protoplasts was adapted from a method described by Guerineau *et al.* (1991). This involved precipitating DNA using PEG and

was thought to facilitate the uptake of DNA by minimising charge repulsion effects.

The Sucrose and $Ca(NO_3)_2$ solutions were sterilised by autoclaving. All other solutions were filter sterilised using 45 μm disposable filters. 0.1 N NaOH was used to adjust the pH of solutions.

Protoplast solution, pH 5.6:

Macerozyme R-10	0.2 mg ml^{-1}
Cellulase Onozuka R-10	1.0 mg ml^{-1}
Mannitol	80.0 mg ml^{-1}
Sucrose	20.0 mg ml^{-1}
MS salts	2.35 mg ml^{-1}

Macerozyme R-10 and Cellulase Onozuka R-10 were purchased from Yakult Honsha Co., Japan. MS salts were developed by Murashige and Skoog (1962).

Protoplast wash solution, pH 5.6:

Mannitol	80.0 mg ml^{-1}
MS salts	2.35 mg ml^{-1}

Sucrose solution:

Sucrose	21 % (w/v)

PEG solution, pH 6.0:

PEG	250.0 mg ml^{-1}
$Ca(NO_3)_2.4H_2O$	23.6 mg ml^{-1}
Mannitol	82.0 mg ml^{-1}
MES	3.9 mg ml^{-1}

PEG average M_r = 6 000, 2-[N-morpholino]ethanesulphonic acid (MES).

Calcium nitrate solution, pH 6.0:

$Ca(NO_3)_2.4H_2O$	65.0 mg ml^{-1}
MES	2.0 mg ml^{-1}

Protoplast recovery medium, pH 5.6:

Mannitol	80.0 mg ml^{-1}
Sucrose	20.0 mg ml^{-1}
MS salts	2.35 mg ml^{-1}

Throughout the procedure the protoplasts were treated with care to minimise rupturing. The lower epidermis was peeled from a detached *N. tabacum* leaf. Pieces of this leaf were placed underside down in a Petri dish containing 30 ml of protoplast solution. The dish was incubated overnight at room temperature in the dark. Protoplasts were loosened from the leaf by gentle agitation and the incubation was continued for a further 30 minutes. Leaf debris was removed by filtering the solution through a 64 μm nitex sieve. The solution was then transferred to a 50 ml centrifuge tube and the protoplasts collected by centrifugation at 100 x*g* for 5 minutes. The supernatant was replaced by 30 ml of protoplast wash solution. The protoplasts were resuspended in the solution and then centrifuged at 100 x*g* for 5 minutes. The washed protoplasts were resuspended in 10 ml of wash solution. 2·5 ml of sucrose solution was pipetted into two 10 ml tubes, each was gently overlaid with 5 ml of the washed protoplasts. The tubes were spun at 150 x*g* for 5 minutes with slow acceleration and deceleration. The protoplasts were collected from the boundary and transferred into a fresh tube. Using a haemocytometer, the protoplast density was calculated. They were then collected by centrifugation at 100 x*g* for 5 minutes and resuspended in wash solution so that the final concentration of protoplasts was between $5x10^6$ and $1x10^7$ ml^{-1}. 200 μl of PEG solution, 20 μg of high quality plasmid DNA and 200 μl of resuspended protoplasts was added to a fresh 10 ml tube. The contents were gently mixed and left at room temperature for exactly 20 minutes. 1 ml of calcium nitrate solution was then added dropwise to the mixture. This was followed by a further 4 ml and the tube was then incubated at room temperature for 10 minutes. Next, the tube was centrifuged at 100 x*g* for 5 minutes and the supernatant was discarded. The protoplasts were resuspended in 5 ml protoplast recovery medium, transferred to a Petri dish and incubated at room temperature overnight. Protoplast were finally harvested by centrifugation at 150 x*g* for 5 minutes, analysis was carried out using methods described in Section 2.13.

2.11.2 Microprojectile Bombardment

The successful delivery of active cDNA into plant cells could be achieved using the Biolistic PDS-1000/He Particle Delivery System (Bio-Rad Laboratories). The system made use of helium gas to build up pressure behind a rupture disc. The shock wave produced following rupture of the disc caused tungsten microparticles, coated in DNA, to be directed at high velocity towards target tissue.

2.11.2.1 Coating Tungsten with DNA

Tungsten microparticles were washed three times using 100 % ethanol

(HPLC grade) and twice using dH_2O. This was carried out by adding 60 µg of tungsten to a microcentrifuge tube. 1 ml of the appropriate wash was then added to the tube. The tungsten was resuspended by vortex and then collected by microcentrifugation at 12 000 x*g* for 2 minutes. The washed microparticles were finally resuspended at a concentration of 60 mg ml^{-1} in dH_2O and stored at $^{-}20^{o}C$. A 50 µl aliquot of tungsten microparticles was transferred to a fresh microcentrifuge tube. ~10 µg of high quality plasmid DNA (~1-2 µg μl^{-1}) was added. The tube was briefly vortexed prior to the addition of 50 µl of $CaCl_2$ (2.5 M), again the tube was vortexed and 20 µl of spermidine (0.1 M, free base) was added. The tube was vortexed for a further 5 minutes, the contents were then collected by microcentrifugation at 10 000 x*g* for 10 seconds. Having discarded the supernatant the pellet was washed using 250 µl of ethanol (HPLC grade). The DNA coated tungsten microparticles were finally resuspended in 60 µl of ethanol.

2.11.2.2 Bombardment of Target Tissue

All microprojectile bombardments were carried out according to the manufacturer's protocol. The procedure was optimised for the bombardment of greenhouse grown *N. tabacum* SR1 target tissue. Each bombardment consisted of 8 µl of resuspended M10 tungsten microparticles coated, in ~1.4 µg of high quality plasmid DNA. The variable parameters were set as follows:

i) Burst pressure of rupture disc = 1 550 lb per square inch
ii) Microcarrier launch assembly set 2 shelves down from the rupture disc assembly.
iii) Target distance set at a further 2 shelves down.
iv) Adjustable nest arranged with the stop screen in the middle position
v) Rupture disc to macrocarrier gap set at 5/8 of an inch
vi) Bombardments were carried out in vacuum of 25 inches of Hg

Target leaf tissue (~2.25 cm^2, unless otherwise stated) was arranged with the underside facing up, in the middle of 0.8 % agarose plates. Following bombardment tissue was placed on moist 3MM paper in a Petri dishe and incubated in a plant growth room until final analysis. Compared with *N. tabacum*, the bombardment of leaf tissue from other plants (including *L. esculentum*) was less successful. The variable parameters were altered, in particular the burst pressure of rupture discs and the size of tungsten microparticles was increased. However, no improvement was observed.

2.12 PLANT TRANSFORMATION

The integration of foreign genes into the plant genome was carried out using the binary vector system of plant transformation. The constructs were cloned into a binary vector, between transferred DNA (T-DNA) border regions. The vector was then conjugated into *Agrobacterium tumefaciens* which already contained a disabled tumour inducing (Ti) plasmid with an active virulence (*vir*) gene. As a result the *vir* gene was able to act *in trans* to transfer the T-DNA region, containing the foreign DNA, into the plant cell.

2.12.1 Use of Antibiotics

Antibiotic	Function	Solvent	Working dilution
Rifampicin	Selection of *A. tumefaciens* strain LBA4404	methanol	50 μg ml^{-1}
Augmentin	Inhibition of *A. tumefaciens* growth	dH_2O	400 μg ml^{-1}
Kanamycin	Selection of transgenic plant tissue	dH_2O	100 μg ml^{-1}

The use of chloramphenicol and kanamycin for the growth of binary vectors in *E. coli* has been described in Section 2.4.2. Stock solutions of augmentin and kanamycin were filter sterilised using 45 μm disposable filters.

2.12.2 Conjugation of Binary Vectors into *A. tumefaciens*

The transfer of binary vectors from *E. coli* into *A. tumefaciens* strain LBA4404 was achieved by triparental mating (van Haute *et al.*, 1983). The helper plasmid pRK2013 (in *E. coli* strain HB101) contained mobilisation (*mob*) and transfer (*tra*) regions. These were able to act on the origin of transfer (*ori T*) and activation site (*bom*) within the binary vector, facilitating the transfer. A 5 ml culture of *Agrobacterium* was grown for 48 hours at 28°C (the Ti plasmid is unstable at higher temperatures) using rifampicin for selection. Independent 5 ml *E.coli* cultures containing either the binary vector or helper plasmid were grown, in the presence of appropriate antibiotics at 37°C overnight. 100 μl of each culture (*Agrobacterium*, binary plasmid and helper plasmid) was pipetted into a microcentrifuge tube and mixed. 10-100 μl of the mix was withdrawn and spread on an NA plate without any antibiotic

selection. The plate was incubated at 28°C for 48 hours and then used to streak a fresh NA plate containing antibiotics to select both *Agrobacterium* and the binary plasmid. Colonies were analysed following incubation for a further 2 days.

Pronase solution:

Pronase	20 μg μl^{-1}
Tris.Cl	10 mM
NaCl	10 mM

Colonies of interest were selected and cultured for 48 hours in NB containing the appropriate antibiotics. 1.5 ml of the culture was transferred to a microcentrifuge tube and the cells collected by microcentrifugation at 12 000 x*g* for 5 minutes. The pellet was resuspended in 300 μl of dH_2O. 100 μl of 10 % N-lauroylsarcosine (sarcosyl) and 150 μl of pronase solution was added to the tube. It was then incubated at 37°C for 1 hour. The mixture was extracted three times using phenol:chloroform and the DNA precipitated using ethanol. The pellet was resuspended in 50 μl of dH_2O and the DNA restricted using suitable enzymes. Agarose gel electrophoresis followed by Southern blot analysis was carried out to transfer DNA on to Hybond N membrane. DNA fragments of interest were identified by probing with a suitable radioactive probe.

2.12.3 Transformation of *N. tabacum*

The following recipes were used to prepare liquid media, solid media also contained 0.85 % agar. All media was sterilised by autoclaving. The recipes and transformation procedure were based on Draper *et al.* (1988).

MSO, pH 5.8:

MS salts	1x
Sucrose	3 % (w/v)

MSD4X2, pH 5.8:

MSO	as above
Naphthalene acetic acid (NAA)	0.1 mg lt^{-1}
6-Benzylaminopurine(6-BAP)	1.0 mg lt^{-1}
(stock solution dissolved in HCl)	

All transformation procedures were carried out in a sterile environment, using sterile equipment. A *N. tabacum* leaf was excised from a healthy plant and surface sterilised, by soaking in a 10 % domestos solution (Lever Industrial Ltd.), for 20 minutes. The leaf was then rinsed using 4 changes of sterile dH_20. The mid-rib was removed from the leaf together with the outside edge. Leaf disc explants ~1.0 cm^2 were then prepared from the leaf, each transformation required ~60 explants. *Agrobacterium* cells harbouring the desired binary plasmid were cultured in 5 ml of NA for 48 hours at 28°C, with appropriate antibiotics for selection. The cells were diluted (1/40) using MSO, they were then poured into a dish into which the explants were placed. Following gentle agitation, to ensure that the *Agrobacterium* made contact with the freshly wounded surfaces, the explants were transferred to antibiotic free MSD4X2 plates (~15 explants per plate). The lids were sealed using cling film and the plates incubated in a plant growth room for 48 hours. Following this incubation it was normal to see *Agrobacterium* growing around the edge of explants. The explants were removed to freshly prepared MSD4X2 plates containing both kanamycin and augmentin. The plates were again sealed and transferred to a plant growth room. Transformed callus normally started to appear at 2-3 weeks post co-cultivation with the *Agrobacterium* cultures, with shoots developing after 4-5 week. Shoots ~1 cm long were transferred to powder rounds containing MSO and the antibiotics kanamycin and augmentin. This encouraged the development of roots from kanamycin resistant, transgenic shoots. As plants grew sub-culturing was necessary. Sections of stem were cut between internodes and transferred to fresh powder rounds containing MSO and kanamycin. Although it was possible to conduct preliminary analysis on plants while in tissue culture they were transferred to soil for further growth and analysis.

2.12.4 Transformation of *L. esculentum*

The procedure followed was supplied by Dr. Arther Pfitzner, Botanisches Institut der Ludwig-Maximilians Universität München, Germany (Charng and Pfitzner, 1994 and personal communication). For discussion see Section 6.3. MS salts and Gamborg's B5 vitamin powder were purchased from Sigma Chemical Company. The pH of the media was adjusted using KOH, solid media also contained 0.85 % (W/V) agar. Media was autoclaved prior to the addition of B5 vitamins, zeatin riboside and 3,5-dimethoxy-4-hydroxyacetophenone (acetosyringone). B5 vitamins and a 1 mg ml^{-1} zeatin riboside stock solution were sterilised by passing through a 45 µm disposable filter, a 10 mM acetosyringone stock solution was prepared in ethanol.

Tomato germination medium, pH 5.7:

MS salts	4.6 mg ml^{-1}

MSOZR, pH 5.4:

MS salts	4.6 mg ml^{-1}
Sucrose	3 % (w/v)
B5 vitamins	1 x
10 mM acetosyringone	0.5 µl ml^{-1}
1 mg ml^{-1} Zeatin riboside	2 µl ml^{-1}

5x Min A salts:

K_2HPO_4	52.2 mg ml^{-1}
KH_2PO_4	22.5 mg ml^{-1}
$(NH_4)_2SO_4$	5.0 mg ml^{-1}
$Na_3C_6H_5O_7.2H_2O$	2.5 mg ml^{-1}

Min A medium:

Prepared from sterile stock solutions.

Min A salts	1x
20 % $MgSO_4$	1 µl ml^{-1}
20 % glucose	10 µl ml^{-1}

Agrobacterium Induction medium, pH 5.4:

MS salts	4.6 mg ml^{-1}
100 mM sodium phosphate, pH 5.4	125 µl ml^{-1}
10 mM acetosyringone	0.5 µl ml^{-1}

Co-cultivation medium, pH 5.7:

MS salts	4.6 mg ml^{-1}
Sucrose	3 % (w/v)
B5 vitamins	1x

Shoot regeneration medium, pH 5.7:

MS salts	4.6 mg ml-1
Sucrose	3 % (w/v)
B5 vitamins	1 x
1 mg ml^{-1} Zeatin riboside	2 µl ml^{-1}

Root regeneration medium, pH 5.7:

MS salts	4.6 mg ml-1
Sucrose	1 % (w/v)
B5 vitamins	1 x

Each independent transformation required 50-60 seedlings. *L. esculentum* seeds were sterilised by submerging in 100 % ethanol for 30 seconds followed by 10 % domestos for 10 minutes. They were then rinsed using three changes of sterile dH_20 and transferred to containers containing tomato germination medium. The containers were stored in the dark for 3-5 days, in which time the majority of seeds germinated. They were then transferred to a plant growth room and grown for a further 8 days. Following this, the cotyledons were carefully removed from the seedlings, each was cut in half and the tip was removed (to reduce damage cotyledons were cut under water). The cotyledon explants were transferred to MSOZR plates (underside down). The plates were sealed and incubated for 24 hours in a plant growth room.

Preparation of the *Agrobacterium* culture began when cotyledons were 6-7 days old. Cells harbouring the desired binary vector were cultured in 5 ml of Min A medium at 28°C for 48 hours, appropriate antibiotics were used for selection. The cells were collected by centrifugation at 4 000 x*g* for 10 minutes and resuspended in 3 ml of *Agrobacterium* induction medium. Following incubation at 28°C for a further 12 hours the cells were again harvested by centrifugation at 4 000 x*g* for 10 minutes. The cells were resuspended in 20 ml of co-cultivation medium.

Following incubation on MSOZR plates, the cotyledon explants were submerged in co-cultivation medium containing the *Agrobacterium* cells. Excess fluid was removed prior to transferring them back to the MSOZR plates. They were then incubated in a plant growth room for 48 hours. Following this, the explants were transferred to plates containing shoot regeneration medium and the antibiotics kanamycin and augmentin. This should have encouraged the growth of callus. *L. esculentum* explants are particularly susceptible to the overgrowth of *Agrobacterium*. As a result, the explants were transferred to fresh plates of selective shoot regeneration medium at weekly intervals. This also reduced the risk of phenolic compounds leaching into the plates. The *L. esculentum* transformation procedure was not continued beyond this point. However, calli ~8 mm^3 should have been transferred to individual containers, and shoots ~1 cm long transferred to root regeneration medium containing kanamycin and augmentin. Sub-culturing plants in tissue culture and soil should have been possible.

2.12.5 Identification of Transgenic Plants

2.12.5.1 Growth on Kanamycin

The ability of callus, shoots and roots to develop in the presence of kanamycin was a strong indication that regenerated T_0 plants were transgenic. However it did not guarantee that the desired insert had been stably integrated into the plant genome. Kanamycin was also used to select future generations of transgenic seedlings. Seeds were sterilised by soaking in 10 % domestos for 10 minutes, they were then rinsed using three changes of sterile dH_2O. Germination was carried out on MSO plates containing kanamycin, in the plant growth room. Although it was normal for all seeds to germinate, only those resistant to kanamycin continued to develop into healthy seedlings. Non-resistant seedlings either bleached or failed to develop past the cotyledon stage. The ratio at which seedling segregated in to kanamycin resistant and non-resistant was used to indicate the copy number of the resistance gene.

2.12.5.2 PCR Analysis

Extraction Buffer:

Tris.Cl, pH 8	100 mM
NaCl	500 mM
EDTA	50 mM
2-mercaptoethanol	0.07 %
SDS	2.8 % (w/v)

Tissue, the size of a microcentrifuge tube lid, was removed from the plant and frozen in liquid nitrogen. It was then homogenised in 250 µl of extraction buffer. Following this the sample was incubated at 65°C for 5 minutes. Potassium acetate (65 µl from a 5 M solution) was then added and the tube was incubated on ice for 5 minutes. Debris was collected by microcentrifugation at 12 000 x*g* for 5 minutes and the supernatant transferred to a fresh tube. 320 µl of propan-2-ol was added to the tube which was then incubated at -20°C for 10 minutes. Precipitated genomic DNA was collected by microcentrifugation at 12 000 x*g* for 10 minutes. The pellet was washed in 70 % ethanol, dried and resuspended in 50 µl dH_2O. 1 µl of the DNA was used in a 50 µl PCR reaction. This was set up according to the method described in Section 2.5.9 using suitable primers and required 30 amplification cycles.

2.13 ANALYSIS OF PLANTS

2.13.1 RNA Dot Blots

Dot blots provide a quick and easy method to assay for the presence of virus (Matthews *et al.*, 1991). They were especially useful when limited plant tissue was available for analysis. Crude RNA extracts were prepared by grinding tissue in liquid nitrogen and then adding dH_2O (~1 ml g^{-1} tissue). 5-10 µl of the extract was then pipetted on to Hybond N membrane which had been moistened in 2x SSC (Section 2.6). Once dry the RNA was fixed to the membrane by exposure to UV light. Preparation of the probe and hybridisation at 42°C was carried out according to methods described in Section 2.7.

2.13.2 Western Immunoblot Analysis

The methods used were based on Draper *et al.* (1988).

2.13.2.1 Antibodies

Primary:

Antibody	Source
Anti TMV-U1 Coat protein (IgG fraction)	Agdia inc., USA.
Anti TMV-U1 126 kDa replicase protein (whole sera)	Prof. William O. Dawson, University of Florida, USA.
Anti PR1a protein (whole sera, also cross reacted with PR1b and PR1c)	R. White, Rothamstead Experimental station, UK.

Secondary:

Conjugate	Source
Alkaline phosphatase	Sigma Chemical Company or Dako-Patts
Horseradish peroxidase	Boehringer Mannheim

2.13.2.2 Protein Extraction

Protein extraction buffer A:
Suitable for the extraction of TMV coat protein.

Potassium phosphate, pH 7.5	35 mM
NaCl	400 mM
2-mercaptoethanol	10 mM

Protein extraction buffer B:
Suitable for the extraction of plant proteins and TMV replicase proteins. A stock solution of Phenylmethylsulphonyl fluoride (PMSF) was prepared in methanol.

Tris·Cl, pH 8	100 mM
EDTA	5 mM
DTT	5 mM
PMSF	4 mM

Plant material was ground in liquid nitrogen and protein extraction buffer (~1 ml g^{-1} tissue) using a mortar and pestle. The extract was transferred into a microcentrifuge tube. Debris was collected by microcentrifugation at 12 000 x*g* for 5 minutes and the supernatant transferred to a fresh tube. In order to ensure that approximately equal amounts of protein were analysed the concentrations were visually compared. To achieve this 5 µl of each sample was pipetted independently on to 3MM paper. Once dry, the samples were stained for 5 minutes by gently agitating in coomassie stain (0.1 % (w/v) Coomassie blue dye, 25 % (w/v) methanol 10 % (w/v) acetic acid). The paper was then soaked in several changes of destain (25 % (w/v) methanol 10 % (w/v) acetic acid), until the background staining had almost disappeared. The intensity of each blue dot was used to estimate the relative concentrations of protein.

2.13.2.3 SDS-PAGE

Denaturing polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using Laemmli mini Bio-Rad II protein gel kits (purchased from Bio-Rad Laboratories). The following recipes were used to prepare one denaturing mini gel.

12 % Resolving gel:

30 % (w/v) acrylamide	3.13 ml
2 % (w/v) bis-acrylamide	375 µl
1 M Tris·Cl, pH 8.8	2.8 ml
10 % (w/v) SDS	75 µl
dH_2O	1.12 ml

Stacking Gel:

30 % (w/v) acrylamide	850 µl
2 % (w/v) bis-acrylamide	100 µl
1 M Tris·Cl, pH 6.9	625 µl
10 % (w/v) SDS	50 µl
dH_2O	3.2 ml

4 x Cracking buffer:

Tris.Cl, pH 6.8	25 mM
SDS	4 % (w/v)
Glycerol	40 %(v/v)
2-mercaptoethanol	20 %(v/v)
Bromophenol blue	0.01 %

Running Buffer:

Tris	25 mM
Glycine	160 mM
SDS	0.3 % (w/v)

The kit, in particular the glass plates, was thoroughly cleaned and assembled according to the manufacture's protocol. A 12 % acrylamide gel solution was prepared. 25 µl of freshly prepared 10 % (w/v) ammonium sulphate and 6 µl of TEMED were added to the solution. It was then immediately pipetted between the glass plates leaving room at the top for the stacking gel (~2 cm). The gel was overlaid with a propanol:dH20 solution (1:1) and left for 1 hour to polymerise. Having removed the propanol:dH_20, the top of gel was rinsed with dH_2O and blotted dry using 3MM paper. Following preparation of the stacking gel 25 µl of 10 % (w/v) ammonium sulphate and 6 µl of TEMED was added and the solution was immediately pipetted between the plates. A comb was inserted, taking care to avoid air bubbles and the gel left to polymerise for 30 minutes. The comb was then removed and the gel assembled in an electrophoresis tank. The buffer chambers were filled with SDS-PAGE running buffer. Air bubbles were removed from the bottom edge of the

plates and the wells were rinsed using running buffer. Protein samples to be resolved were suspended in 1x cracking buffer (up to a total volume of 40 µl) and denatured, by heating at ~90°C for 2 minutes. The samples were then immediately loaded into the wells. At the same time molecular weight markers (SDS-7 or SDS-6H, Sigma Chemical Company) were loaded to indicate the size of proteins. Electrophoresis was carried out at 150 v and the blue dye in the cracking buffer was used to monitor progress.

2.13.2.4 Transfer of Protein to Membrane

This was carried out using a semi dry Millipore-SDE system (Millipore).

Anode Buffer 1:

Tris·Cl, pH 10.4	300 mM
Methanol	10 % (v/v)

Anode Buffer 2:

Tris.Cl, pH 10.4	25 mM
Methanol	10 % (v/v)

Cathode Buffer:

Tris.Cl, pH 9.4	25 mM
6-aminohexanoic acid	40 mM
Methanol	20 % (v/v)

Three pieces of 3MM paper and one piece of membrane (either Immobilon-P from Millipore or Nitrocellulose extra from Sartorius AG, Germany) were cut to the size of the gel. The blot was then assembled in the following order: i) Cathode ii) 3MM soaked in cathode buffer iii) gel iv) nitrocellulose membrane prepared according to the manufacturer's instructions v) 3MM soaked in anode buffer 2 vi) 3MM soaked in anode buffer 1 vii) anode. The transfer of protein from the gel on to the membrane was achieved using a current of 150 mA (per mini gel) for 1 hour. Once transferred the gel membrane was removed and stained using ponceau stain (0.2 % (w/v) Ponceau stain in 3 % (w/v) TCA) for ~5 minutes. Excess stain was removed by rinsing in dH_2O. The stain made it possible to visualise proteins and to mark the position of molecular weight markers. It was eventually removed by washing in TBS (Section 2.13.2.5).

2.13.2.5 Immuno-Detection of Protein

Blocking Solution:

Polyoxyethylene-sorbitan monolaurate (Tween 20).

Tris.Cl, pH 9.0	100 mM
NaCl	150 mM
Marvel (Cadbury)	% (w/v)
Tween 20	0.05 % (v/v)

Antibody Solution: As for blocking solution, except it contained only 1% (w/v) Marvel. Antibody was then added, according to the supplier's instruction.

Tris buffered saline (TBS):

Tris.Cl, pH 7.4	50 mM
NaCl	200 mM

TBS-Tween:

TBS	1x
Tween 20	0.1 %

Following electroblotting, the membrane was incubated in 50 ml of blocking solution for 1 hour. This was carried out using a rotary shaker and at room temperature. It was then transferred to a sealed bag containing 5 ml of antibody solution and the appropriate primary antibody. Incubation was carried out either at room temperature for 4 hours or at 4°C overnight. Unbound antibody was removed by washing 5 times (each for 10 minutes) in 100 ml of TBS-Tween. The membrane was then incubated for one hour, at room temperature in 5 ml of antibody solution, containing the appropriate secondary antibody. Again excess antibody was removed by washing 5 times in 100 ml of TBS-Tween.

2.13.2.6 Visualisation

Alkaline phosphatase conjugated secondary antibodies

BCIP buffer:

Tris.Cl, pH 9.5	100 mM
$MgCl_2$	1 mM

BCIP stock: 50 mg ml^{-1} of 5-bromo-4-chloro-indolyl phosphate (BCIP), in dimethyl formamide.

NBT stock: 30 mg ml^{-1} of nitroblue tetrazolium (NBT), in dimethyl formamide

50 µl of BCIP stock solution and 50 µl of NBT stock solution was added to 5 ml of BCIP buffer. The mixture was immediately poured on to the washed membrane which had been positioned protein side up. If visualisation was expected to take >5 minutes development was carried out in the dark. In order to stop the reaction the membrane was rinsed using copious amounts of water.

Horseradish peroxidase conjugated secondary antibodies

Chemoluminescent detection was estimated to be 10 times more sensitive than other available techniques. It was carried out using a BM Chemiluminescent kit (Boehringer Mannheim) according to the manufacturer's protocol.

2.13.3 Assaying for Luciferase Activity

All solution were freshly prepared, from stocks, at the time of assay.

Luciferase extraction buffer:

Potassium phosphate, pH 7.5	100 mM
DTT	1 mM

Luciferin solution:

Potassium phosphate, pH 7.5	100 mM
Luciferin	0.05 mM

ATP solution:

Potassium phosphate, pH 7.5	5 mM
HEPES, pH 7.5	50 mM
$MgCl_2$	20 mM
ATP	10 mM

Luciferin was purchased from Sigma Chemical company. Samples were ground for 1 minute in luciferase extraction buffer (~1 ml g^{-1} tissue) and then

transferred into a microcentrifuge tube. Plant debris was pelleted by centrifugation at 12 000 x*g* for 5 minutes. Using a Clinilumat LB 9502 luminometer (EG & G Berthold), the light emitted from 50 µl of sample in the presence of 100 µl luciferin solution and 100 µl ATP solution was measured over a 10 second period. The reading in light units (LU) was compared to background activity measured over a 1 second period.

2.13.4 Assaying for GUS Activity

2.13.4.1 Quantitative Fluorimetric Analysis

GUS Extraction Buffer:

Sodium phosphate, pH 7.0	50 mM
EDTA	10 mM
Triton X-100	0.1 % (v/v)
Sarcosyl	0.1 % (w/v)
2-mercaptoethanol	0.07 %

MUG solution: 1 mM Methylumbelliferyl β-Glucuronide (MUG), purchased from Sigma Chemical Company, in GUS extraction buffer.

Stop solution:

Na_2CO_3	200 mM

Samples were ground for 1 minute in GUS extraction buffer (~1 ml g^{-1} tissue) and then transferred to a microcentrifuge tube. Debris was pelleted by centrifugation at 12 000 x*g* for 5 minutes. Alternatively extracts in luciferase extraction buffer were used for the analysis. 125 µl of extract was mixed with 375 µl of MUG solution and incubated at 37°C for 10 minutes. 100 µl of the reaction was withdrawn from the tube and transferred to 100 µl stop solution which had been pipetted into the well of a black microtitre plate. Fluorescent units (FU) were measured using a Microfluor reader (Dynatech Laboratories). Fluorescence occurred when the β-glucuronidase (GUS) enzyme catalysed the conversion of MUG to 4-methyl umbelliferone (4-MU). Future time points were dependent on the initial, time (t) = 0, reading. If FU <500 three further reading were taken at 5-15 minute time intervals. Where FU >500 the sample was diluted with GUS extraction buffer and a new reaction set up. Using FU readings taken at 4 time points the rate of increase in fluorimetric activity (FU min^{-1}) was calculated. Dilutions of 4-MU were

used to produce a linear calibration graph. This allowed the FU min^{-1} readings to be converted into pMol 4-MU min^{-1}.

2.13.4.2 Qualitative Histochemical Analysis

X-GLUC stock: 10 mM 5-bromo-4-chloro-3-indoyl-β-glucorinide (X-GLUC), purchased from Melford Laboratories, in dimethyl formamide.

X-GLUC solution:

Potassium phosphate, pH 7.0	10 mM
EDTA	1 mM
X-GLUC	1 mM
$K_4Fe(CN)_6.3H_2O$	0.5 mM
$K_3Fe(CN)_6$	0.5 mM

Samples were submerged in X-GLUC solution and placed in a vacuum desiccator for 10 minutes. They were then incubated overnight at 37°C. In order to visualise the blue precipitate, the sample was destained in 100 % industrial methylated spirits overnight, for longer storage tissue samples were transferred into 70 % industrial methylated spirits.

2.13.5 Calculating Protein Concentration

Bradford's Reagent (Bradford, 1976):

Coomassie blue G-250	0.1 mg ml^{-1}
Perchloric acid	2 % (v/v)

The solution was stirred overnight, filtered and then stored in the dark.

20 µl of the sample (in either GUS or luciferase extraction buffer) was pipetted into the well of a clear microtitre plate. 180 µl of Bradford's reagent was added to the well. The concentration of protein was measured using a Dynatech MR 5000 spectrophotometer. The machine was programmed to measure the A_{630} of each sample in duplicate and calculate an average. This was compared to BSA standards giving the final protein concentration in mg ml^{-1}.

CHAPTER 3

INVESTIGATING THE INFECTIVITY OF A CaMV 35S PROMOTER DRIVEN cDNA CLONE OF TMV

3.1 INTRODUCTION

Studies of TMV have been aided by the stability and prevalence of the virus, as well as the ease at which host plants can be manually inoculated with the many different strains. However, perhaps the most important breakthrough in the study of viruses with RNA genomes has been the application of the enzyme reverse transcriptase (Section 1.4.1). This has enabled full length cDNA clones to be generated, being stable DNA is more suited to molecular techniques. As a result, it is possible to make precise alterations to the TMV genome. Infection normally requires that the artificial cDNAs are fused to suitable promoters capable of directing transcription to produce RNA. Initially transcription was carried out *in vitro* following the carefull fusion of cDNA clones to promoters active in bacteria (Section 1.4.2). The production of capped transcripts was shown to increase infectivity (Ahlquist *et al.*, 1984; Dawson *et al.*, 1986; Meshi *et al.*, 1986; Janda *et al.*, 1987), as was the reconstitution of RNA into virus particles prior to inoculation (Dawson *et al.*, 1986; Meshi *et al.*, 1986). The *in vitro* transcription of manipulated cDNA clones fused to bacteriophage promoters has been widely used to study TMV. However, it was recognised that the fusion of viral cDNAs to promoters active in plants also offered many advantages (Section 1.4.3). In particular transcription *in vivo* would allow viral cDNA constructs to be stably integrated into the plant genome. In order to achieve this many viral cDNA clones have been directly fused to the 35S promoter (Guilley *et al.*, 1982; Odell *et al.*, 1985) from CaMV. *N. tabacum* Samsun plants have been successfully transformed with a 35S promoter driven full length cDNA clone of TMV (Yamaya *et al.*, 1988). Both viral RNA or 35S driven cDNAs are suitable for transient assay procedures, including the infection of protoplasts and microprojectile bombardment experiments. The production of 35S-cDNA clones which could also be manually inoculated on to plants was an attractive goal. With this aim a number of infectious constructs have now been built (Section 1.4.3). Unfortunately, they tended not to demonstrate the normal host range of the virus concerned, following the manual inoculation of leaves. For example, a 35S-TMV-L cDNA was highly infectious on *Chenopodium quinoa*, yet failed to infect its normal host *L. esculentum* (Weber *et al.*, 1992).

Many of the constructs described in this thesis are based on a 35S promoter driven cDNA clone of TMV-U1. It was essential to establish the ability of the intact construct to infect the desired plants prior to molecular manipulation. For interest, this initially involved the manual inoculation of plants. More importantly for the needs of this project, infectivity via the inoculation of protoplasts, microprojectile bombardment of detached leaves and plant transformation were investigated.

3.2 RESULTS

3.2.1 The Design of p35STMVR

Through a collaboration with Dr. Richard S. Nelson (The Samuel Roberts Noble Foundation, Plant Biology Division, Oklahoma, USA) we received a full length infectious cDNA clone of TMV-U1 fused to the 35S promoter (Figure 3.1). Despite modern communication systems confusion occurred over the name of the construct. As a result while the construct is to be published as p35SU1R throughout this thesis it will be referred to as p35STMVR. Full details of the construction of p35STMVR in the plasmid vector pAlter-1 are described by Shintaku *et al.* (1996). Only brief details are given below in order to demonstrate that constraints governing the infectivity of such constructs (discussed in Section 1.4 and reviewed by Boyer and Haenni, 1994) had been observed.

A modified 35S promoter, with duplicated enhancer region, was taken from the plant expression vector pRTL2 (Restrepo *et al.*, 1990). This was linked to a full length infectious cDNA clone of TMV-U1 previously described as U3/12-4 (Holt and Beachy, 1991). It was important to ensure that the promoter was correctly fused to the TMV cDNA so that transcription began with the first nucleotide of the TMV genome (Dawson *et al.*, 1986; Janda *et al.*, 1987). This was achieved using a recombinant PCR protocol (Higuchi, 1989) to create a correctly fused product consisting of the 3' end of the 35S promoter and the 5' end of the TMV cDNA. The remaining 35S promoter and TMV cDNA were added in a series of ligations, using fragments generated by cleavage at existing restriction enzyme sites. Although not essential, decreasing the number of 3' non-viral nucleotides has been found to increase infectivity (Meshi *et al.*, 1986; Janda *et al.*, 1987; Turpen *et al.*, 1993). A hammer-head class ribozyme cassette (reviewed by Symons, 1991) was introduced downstream of the TMV genome. This was designed to catalyse the self cleavage of transcripts, leaving just 4 non-TMV nucleotides.

3.2.2 Transfer of the Infectious Clone to Other Vectors

The described infectious TMV cDNA construct was used extensively throughout this project. In order to gain additional restriction enzyme sites for cloning, it was necessary to transfer it from pAlter-1 to pcDNA II (cloning techniques are described in Section 2.5). p35STMVR was digested using *Pst* I and the entire 35S-cDNA-ribozyme cassette was religated into *Pst* I digested, dephosphorylated

pcDNA II. The new construct was designated pC-35STMVR (Figure 3.1). Restriction enzyme digests were used in order to orientate the insert within the pcDNA II vector (Figure 3.2). They revealed that the 5' end of the 35S promoter was closest to the M13 Forward primer site (Figure 3.1). This cloning step meant that the ampicillin resistance gene could be used for bacterial selection, as opposed to the tetracycline resistance gene in pAlter-1. The use of tetracycline for bacterial selection had on occasions been a problem. Prior to plant transformation it was necessary to transfer the construct from pcDNA II to the binary vector pBin 19. pC-35STMVR was digested using *Xho* I and *Kpn* I, the 35S-cDNA insert was religated into *Sal* I and *Kpn* I digested pBin 19 (*Xho* I and *Sal* I being compatible), the resulting construct pB-35STMV consisted of the 35S promoter fused to the TMV cDNA, but without the ribozyme (Figure 3.1).

3.2.3 Manual Inoculation of Plants

On being given the construct p35STMVR we were warned that it did not appear to infect some plants known to be hosts of TMV-U1. Similar observations have been made about other 35S-viral cDNA constructs (Section 1.4.3). p35STMVR had been shown to induce systemic necrosis on *N. benthamiana* and lesions on *Chenopodium amaranticolor* following the manual inoculation of plants. The symptoms were indistinguishable from those caused by inoculating the plants with purified TMV-U1 particles. However, both *N. tabacum* Xanthi and *N. tabacum* Xanthi nc remained symptomless following manual inoculation with 25 µg of p35STMVR. Sap from p35STMVR infected *N. benthamiana* and *C. amaranticolor* was highly infectious when manually inoculated to *N. tabacum* Xanthi plants. Further manual inoculation experiments were carried out in Dr. R. S. Nelson's laboratory to investigate the effect of the ribozyme. Local lesion assays on *C. amaranticolour* were used to assess the infectivity of p35STMVR and a similar construct, p35SU1AR, which had the ribozyme inserted in the reverse orientation. The antisense ribozyme construct was shown to be only ~7 % as infectious as p35STMVR (results presented by Dagless *et al.*, 1997).

Using p35STMVR and pC-35STMVR the manual inoculation of plants (Section 2.10.1) to be used throughout this project was investigated further. *N. benthamiana* was used as a positive control. Using 10 µg of high quality purified plasmid (Section 2.5.1.5) it was confirmed that both p35STMVR and pC-35STMVR were highly infectious when manually inoculated on to *N. benthamiana.* Characteristic wilting and necrosis started to develop at 3 days post inoculation and led to death of the plants by 10-14 days post inoculation. Tissue from upper uninoculated leaves was removed at 5 days post inoculation and used to confirm the presence of TMV by RNA dot blot and

western immunoblot analysis (see Sections 2.13.1 and 2.13.2 respectively). The results are presented in Figures 3.3 and 3.4. The dot blot was probed with the ~3.6 kb *Hind* III fragment isolated from p35STMVR (nucleotides 1446-5080 of the TMV-U1 genome). Western immunoblots were probed with antibody raised to TMV coat protein and visualised using an alkaline phosphatase conjugated secondary antibody. Although as little as 2 μg of pC-35STMVR has been used to infect *N. benthamiana* (result not shown) the inoculation of 10 μg consistently resulted in infection. Inoculation experiments were carried out using *N. tabacum* SR1, *L. esculentum* GCR 26 (+/+) and *L. esculentum* GCR 237 (*Tm-1/Tm-1*). It was assumed that the presence of 2 copies of the *Tm-1* resistance gene in the latter should greatly reduce the replication of TMV (Section 1.9.2). Duplicate plants were inoculated using 10 μg of the purified high quality plasmid p35STMVR. At the same time similar control plants were inoculated with ~10 μg of purified TMV particles (Section 2.10.2). At 12 days post inoculation mosaic symptoms were apparent on the systemically infected leaves of TMV inoculated *N. tabacum* SR1 plants. Symptoms were less apparent on *L. esculentum* GCR 26 (+/+), despite reports that TMV-U1 should induce mosaic symptoms on *L. esculentum* (Fraser *et al.*, 1986). Systemic leaf samples taken at 12 and 24 days post inoculation and were used to prepare protein samples. Approximately equal amounts of protein (estimated by the method described in Section 2.13.2.2) were used for western immunoblot analysis and filters were probed with antibody raised to TMV coat protein. The results have been presented in Figure 3.5 and 3.6. TMV coat protein was not detected in plants inoculated with p35STMVR, indicating that the procedure had failed. Coat protein was detected in control *N. tabacum* SR1 and *L. esculentum* GCR 26 (+/+) plants following inoculation with purified virus particles. The absence of TMV coat protein in the *L. esculentum* GCR 237 (*Tm-1/Tm-1*) plants demonstrates the effectiveness of the *Tm-1* resistance gene.

Further attempts were made to manually inoculate *N. tabacum* SR1 plants with either p35STMVR or pC-35STMVR. Plants were kept for at least 20 days post inoculation before final RNA dot blot analysis. Filters were probed with an ~3.6 kb *Hind* III fragment isolated from pC-35STMVR. Changing the volume of dH_2O in which plasmid was suspended, from 10 μl to 100 μl, prior to inoculation appeared to have no effect. *N. benthamiana* was consistently infected by 10 μg of p35STMVR or pC-35STMVR irrespective of the volume in which they were suspended. Manual inoculation was normally carried out on leaves that were 1/2-3/4 fully expanded. Attempts to inoculate younger *N. tabacum* SR1 leaves and even the shoot apex also failed (results not presented). It has been suggested that excision of 35S-viral cDNA constructs from their plasmid vectors can overcome problems with infectivity (Neeleman *et al.*, 1993; Ding *et al.*, 1995). In order to investigate this the 35S-TMV

cDNA-ribozyme construct was excised from pcDNA II using flanking *Pst* 1 sites. The digested plasmid (both vector and insert) was extracted using phenol:chloroform, precipitated using ethanol and resuspended in dH_2O at a concentration of ~1 ug ul^{-1}. Using 10 µg of either digested or circular plasmid *N. tabacum* SR1 and *N. benthamiana* plants were inoculated in duplicate. At 7 days post inoculation samples were taken from the inoculated leaves and used for RNA dot blot analysis. The result, presented in Figure 3.7, showed that both circular and linear plasmids were infectious on *N. benthamiana*. Even excised plasmid remained uninfectious to *N. tabacum* SR1. Again using *Pst* I digested pC-35STMVR, both 10 µg and 30 µg was used to inoculate *N. tabacum* SR1 and *N. tabacum* Samsun *NN* plants. The latter cultivar was homozygous for the *N* resistance gene which interacts with TMV to induce the HR (Sections 1.9.1; 1.9.4). As a result TMV is confined to the region immediately surrounding lesions. At 12 days post inoculation with 10 µg of digested pC-35STMVR a single lesion was observed on the inoculated leaf of a *N. tabacum* Samsun *NN* plant. A RNA dot blot was used to identify the presence of TMV in the area surrounding this lesion (see Figure 3.8). When the experiment was repeated no lesions developed on *N. tabacum* Samsun *NN* plants. Further attempts were made to inoculate *N. tabacum* SR1 with digested pC-35STMVR (vector and insert) and also the purified 35S-TMV cDNA-ribozyme insert alone. During these investigations one *N. tabacum* SR1 plant inoculated with purified insert started to slowly develop symptoms at ~15 days post inoculation. RNA dots blots of samples taken from upper uninoculated leaves failed to detect TMV at 7 days post inoculation, although they showed the virus to be present at 28 days post inoculation (results not presented). In total over 50 attempts were made to inoculate *N. tabacum* plants with either p35STMVR or pC-35STMVR, this resulted in only 2 infections (equivalent to an ~4 % rate of infection).

3.2.4 Infection of Protoplasts

Protoplasts can be useful for the study of virus infections at the cellular level, as long as the limitations of these isolated cells are recognised (Takebe, 1975). It was decided to investigate the ability of p35STMVR to infect freshly isolated leaf mesophyll cell protoplasts from *N. tabacum* SR1. Approximately $1x10^6$ protoplasts were inoculated with 10 µg of purified p35STMVR and incubated overnight (the procedure is described in Section 2.11.1). Half of the extract was used to prepare a protein sample for western immunoblot analysis, the other half was used to inoculate a healthy *N. tabacum* SR1 plant. Symptoms developed on the inoculated plant by 7 days post inoculation and tissue from an upper uninoculated leaf was used to prepare a second protein sample. Following SDS-PAGE protein from the two samples was probed with

antibody raised to the TMV-U1 coat protein and visualised using an alkaline phosphatase conjugated secondary antibody (Figure 3.9). Only a faint TMV coat protein signal was detected in the sample prepared from the protoplast extract, this may have been due to contamination occurring as the gel was loaded. A strong signal was present in the sample prepared from the inoculated *N. tabacum* SR1 plant indicating that the protoplast extract was highly infectious.

3.2.5 Microprojectile Bombardment Experiments

Microprojectile bombardment has proved to be a valuable method by which both DNA and RNA can be delivered into cells. It was decided to investigate whether p35STMVR could induce a TMV infection, following microprojectile bombardment into detached *N. tabacum* SR1 leaves. The initial experiment was conducted in a similar manner to the protoplast experiment described in Section 3.2.4. Two leaf samples ~2.25 cm^2 were removed from a young *N. tabacum* SR1 leaf (<8 cm long). The underside of each leaf sample was bombarded with ~1.4 μg of purified p35STMVR according to the protocol described in Section 2.11.2. Following incubation for 24 hours, sap from one of the leaf samples was used to prepare protein for western immunoblot analysis. Sap from the second leaf sample was used to inoculate a healthy *N. tabacum* SR1 plant. Mosaic symptoms typical of a TMV infection developed on upper uninoculated leaves. Tissue from one of these leaves was removed at 7 days post inoculation and used to prepare a second protein sample. Following western immunoblot analysis TMV coat protein was detected in both protein samples (Figure 3.9). The signal was much stronger in the sample prepared from the inoculated plant. Although the faint band in the bombarded sample may have indicated that significant levels of TMV were present by 24 hours post bombardment, it could also have been a result of contamination occurring as the gel was loaded.

Encouraged by the described protoplast and microprojectile bombardment experiments we chose to investigate microprojectile bombardment further. The decision was based on the ease of the bombardment protocol and the ability to incubate leaves for 5-7 days post bombardment, without visible signs of deterioration. The protoplast protocol was more time consuming (in particular peeling the lower epidermis from leaves required both patience and skill) and viability of protoplasts deteriorated with continued storage. It was decided to bombard *N. benthamiana*, *N. tabacum* SR1, *L. esculentum* GCR 26 (+/+) and *L. esculentum* GCR 237 (*Tm-1/Tm-1*) with the construct p35STMVR. In an attempt to determine the success of bombarding the different leaf types, each bombardment consisted of purified p35STMVR and a control 35S-luciferase construct, designated pRTS2-LUC (Turner *et al.*, 1994).

Unfortunately due to the instability of the luciferase enzyme, luciferase assays had to be carried out by 24 hours post bombardment. It was planned to store leaf tissue for longer periods. The results produced from assaying part of each leaf sample after 24 hours were therefore only used to estimate the success of bombardments. As described in Section 2.11.2.2 the microprojectile bombardment procedure was optimised for young green house grown *N. tabacum* SR1 leaves (<8 cm long). It was presumed that these young leaves possessing compact cells, small vacuoles and a less developed cuticle would be most suited to the procedure. Although bombardments into *N. benthamiana* were comparable with *N. tabacum* SR1, bombardments into *L. esculentum* were less effective. Attempts were made to further optimise the procedure but no improvement was observed. It was therefore important to carefully select young *L. esculentum* leaves for the microprojectile bombardment procedure.

Approximately 1.15 µg of p35STMVR and ~0.25 µg of pRTS2-LUC were independently bombarded into 6 samples cut from each of the following plants - *N. benthamiana*, *N. tabacum* SR1, *L. esculentum* GCR 26 (+/+) and *L. esculentum* GCR 237 (*Tm-1/Tm-1*). At 24 hours post bombardment ~1/6 of each leaf sample was removed and used to approximate the luciferase activity for the plant type (luciferase assays are described in Section 2.13.3). The luciferase activities were *N. benthamiana* 327 080 LU; *N. tabacum* SR1 2 608 930 LU; *L. esculentum* GCR 26 (+/+) 84 730 LU; *L. esculentum* GCR 237 (*Tm-1/Tm-1*) 46 290 LU. Leaf samples were incubated for a further 5 days and then assayed for the presence of TMV. Protein samples were prepared and used for western immunoblots. These were probed with antibody raised to the TMV-U1 coat protein (Figure 3.10). Coat protein was detected in *N. benthamiana*, *N. tabacum* SR1 and *L. esculentum* GCR 26 (+/+) leaf samples following microprojectile bombardment with p35STMVR. As expected coat protein was not detected in the resistant *L. esculentum* GCR 237 (*Tm-1/Tm-1*) leaf sample. These results were confirmed by an RNA dot blot probed with an ~3.6 kb *Hind* III fragment isolated from p35STMVR. TMV RNA was detected in all bombarded plant types except *L. esculentum* GCR 237 (*Tm-1/Tm-1*) (Figure 3.11). Further confirmation that p35STMVR was able to induce a TMV infection was gained by examining samples using transmission electron microscopy. Full length TMV-like particles were identified in *N. benthamiana*, *N. tabacum* SR1 and *L. esculentum* GCR 26 (+/+) samples following microprojectile bombardment with p35STMVR (Figure 3.12). Examination of many fields of view for an equivalent period of time resulted in only one TMV-like particle being identified in the bombarded sample from *L. esculentum* GCR 237 (*Tm-1/Tm-1*). Sap from bombarded tissue was used to manually inoculate healthy *N. tabacum* SR1 plants. Symptoms developed within 10 days of inoculation. An RNA dot blot using tissue taken from upper uninoculated

leaves was used to confirm the presence of TMV RNA. The result (presented in Figure 3.13) indicated that a TMV infection had developed in all four inoculated *N. tabacum* SR1 plants. Symptoms on the *N. tabacum* SR1 plant that had been inoculated with sap from p35STMVR bombarded *L. esculentum* GCR 237 (*Tm-1/Tm-1*) were the least obvious at 10 days post inoculation. In addition less TMV RNA was detected in this plant following RNA dot blot analysis (Figure 3.11). The result confirmed previous observations that TMV is capable of limited multiplication in hosts homozygous for the *Tm-1* gene (Section 1.9.2). The bombardment experiments were repeated in order to confirm the results (results not presented). Again TMV RNA was detected in *N. benthamiana, N. tabacum* SR1 and *L. esculentum* GCR 26 (+/+), yet remained undetectable in the *L. esculentum* GCR 237 (*Tm-1/Tm-1*) leaf samples. This was despite the luciferase activity at 24 hours post bombardment being 72 040 LU for the *L. esculentum* GCR 237 (*Tm-1/Tm-1*) sample and only 9 140 LU for the *L. esculentum* GCR 26 (+/+) sample. Leaf tissue from *P. sativum*, a non-host of TMV, was bombarded with ~1.14 µg p35STMVR and ~0.25 µg of pRTS2-LUC. The luciferase activity, measured at 24 hours post bombardment, was 162 580 LU. However, an RNA dot blot failed to detect TMV RNA in the *P. sativum* tissue at 6 days post bombardment (result not presented).

Experiments were designed to determine the ability of pC-35STMVR to infect *N. tabacum* SR1 plants by microprojectile bombardment. At the same time it was decided to investigate the role of the ribozyme further and to determine the stability of plasmid DNA, following either linearisation or excision of the 35S-TMV cDNA insert from the pcDNA II vector. p35STMVR and pC-35STMVR should both have undergone post transcriptional ribozyme cleavage downstream of the 3' end of the TMV genome. Microprojectile bombardment was used to compare the infectivity of these constructs with pC-35STMVR that had been cleaved, using restriction enzymes, prior to bombardment. Three enzymes were used to digest pC-35STMVR *Pst* 1, *Kpn* 1 and *Xho* 1. Using *Pst* 1 the entire 35S promoter-virus-ribozyme construct was excised from pcDNA II. This ensured that additional 3' non-TMV nucleotides were limited to some extent irrespective of whether the ribozyme was correctly functioning. The restriction enzyme *Kpn* 1 was used to cleave the construct between the 3' end of the TMV genome and the ribozyme, allowing only one additional 3' non-viral nucleotide to be transcribed. In order to determine the stability of the construct when cleaved upstream of the 35S promoter it was digested using *Xho* 1. The ribozyme was required to cleave transcripts downstream of the TMV genome leaving four non-TMV nucleotides. Following restriction enzyme digestion, DNA was purified by phenol:chloroform extraction and precipitated using ethanol. The DNA was

then resuspended at a concentration of ~1 μg μl^{-1} using dH_20. Microprojectile bombardment was used to deliver ~1.15 μg of each circular or digested construct into six *N. tabacum* SR1 leaf samples (~2.25 cm^2). Each bombardment also contained ~0.25 μg of pRTS2-LUC, which acted as a positive control, to estimate the success of bombardments. Following incubation for 24 hours luciferase assays were carried out using 1/6 of each bombarded leaf sample. The luciferase activities were determined to be circular p35STMVR 1 811 690 LU; circular pC-35STMVR 616 520 LU; *Pst* I digested pC-35STMVR 1 235 290 LU; *Kpn* digested pC-35STMVR 1 503 050 LU and *Xho* I digested PC-35STMVR 1 323 310 LU. Two leaf samples from each type of bombarded tissue were removed for analysis at 2, 4 and 6 days post bombardment. The samples were used for western immunoblot analysis and RNA dot blot analysis in order to determine levels of coat protein and TMV RNA respectively. Following SDS-PAGE, western immunoblots were probed with antibody raised to TMV-U1 coat protein. RNA dot blots were probed with the 3.6 kb *Hind* III fragment isolated from pC-35STMVR. The results, presented in Figures 3.14 and 3.15, demonstrated that TMV was detectable by 4 days post bombardment irrespective of the type of construct. In each case the level of TMV coat protein and RNA increased up to 6 days post bombardment. The direct comparison of signal intensities following bombardment with the different types of constructs was not possible. This was due to variations in the bombardment procedure which could only be estimated by the luciferase assays. However, the results suggest that the ribozyme was adequately functioning to cleave RNA transcripts. Also the stability of the DNA did not appear to be compromised by linearisation or excision of the 35S promoter-virus-ribozyme construct prior to bombardment.

3.2.6 Transformation of *N. tabacum* SR1 Using pB-35STMV

The plasmid pBin 19 is designed to be used in conjunction with some strains of *Agrobacterium* for the binary vector system of plant transformation. The construct pB-35STMV consisted of the 35S-TMV cDNA construct but did not include the ribozyme sequence (Figure 3.1), cloned into the T-DNA region of pBin 19 adjacent to the gene for kanamycin resistance. The procedure for the transformation and regeneration of *N. tabacum* is described in Section 2.12. *N. tabacum* SR1 explants were co-cultivated for 48 hour with *Agrobacterium* strain LBA4404 harbouring the plasmid pB-35STMV. The explants were then transferred to MSD4X2 plates containing kanamycin to select transgenic cells and augmentin to inhibit further growth of *Agrobacterium*. Callus started to develop by 4 weeks post co-cultivation and was followed by the appearance of shoots within 6 weeks. Following transfer into jars containing MSO media seven shoots continued to grow and kanamycin resistant roots

developed. These plants were numbered and given the prefix "U". All regenerated plants exhibited severe mosaic symptoms which were characteristic of TMV infections. The symptoms indicated that the complete 35S-TMV cDNA construct from pB-35STMV had been successfully integrated into the plant genomes. However infection could also have occurred following the infection of cells on the same leaf disc as a regenerated plant. In this situation TMV would have been expected to spread throughout the leaf disc infecting all attached callus and shoots. Tissue samples taken from the regenerated plants U1-U7 were used for RNA dot blot analysis. At the same time tissue was sampled from the centre of two explants. RNA dot blot analysis was carried out using a probe made from an ~3.6 kb *Hind* III fragment isolated pC-35STMVR. The result, presented in Figure 3.16, indicated the presence of TMV RNA in all samples. PCR analysis was carried out to determine whether the regenerated TMV infected plants contained the 35S-TMV cDNA insert (Section 2.12.5.2). Two primers had been designed to amplify a region from the TMV-U1 replicase genes. Primer 5'REP, 5' TGGCGAAACTCAGAACTCTGCG 3', was identical to nucleotides 2485-2506 of the TMV-U1 genome, primer 3'REP, 5' AAACATCAGAGTATGTCTCGCC 3', was complementary to nucleotides 3198-3219. The combined use of these primers should have amplified a 735 bp fragment. PCR analysis suggested that all plant lines with the exception of U3 were transgenic (Figure 3.17). These plants remained in tissue culture prior to being discarded, a photograph of plant U1 is shown in Figure 3.21A.

A second transformation was carried out using *N. tabacum* SR1 explants and the construct pB-35STMVR. Only 1 plant was regenerated and designated U8. The plant displayed severe mosaic symptoms and was transferred to soil to allow leaf development prior to analysis. PCR analysis using primers 5' REP and 3' REP indicated that the 35S-TMV cDNA construct had been stably integrated into the plant genome (Figure 3.18). We were interested in the fact that mosaic symptoms were displayed on the line U plants despite the fact that every cell should be producing TMV RNA. It was attempted to separately sample equal amounts of light and dark green leaf tissue from the plant U8. The samples were used for western immunoblot analysis and RNA dot blot analysis . The western immunoblot was probed with antibody raised to TMV-U1 coat protein and visualised using alkaline phosphatase conjugated secondary antibody, see Figure 3.19. RNA dot blot analysis was carried out using a probe prepared from an ~3.6 kb *Hind* III fragment isolated from pC-35STMVR, the result is presented in Figure 3.20. TMV RNA and coat protein was detected in samples prepared from both dark green and light green tissue, although the signals appeared to be stronger in the samples prepared from light green tissue. Photographs of plant U8 which clearly demonstrate the mosaic symptoms as well as the distorted appearance of

leaves are shown in Figure 3.21 (B and C).

3.3 DISCUSSION

Recently attempts have been made to build infectious cDNA clones of RNA viruses which can be transcribed *in vivo*. These would complement pre-existing viral cDNAs which require *in vitro* transcription prior to the inoculation of plants. It was intended to use a 35S driven cDNA clone of TMV-U1, described in Section 3.2.1, to build constructs for the investigations described in Chapters 4, 5, 6 and 7. It was therefore essential to establish the ability of the clone to induce a TMV infection in the host plants which we planned to study. Three similar constructs have been described. Two of the constructs should have contained identical cDNA clones of TMV directly fused to the 35S promoter in order to drive transcription. A ribozyme sequence was inserted at the 3' end of the TMV genome in order to catalyse self cleavage of the transcripts. The constructs differed in that p35STMVR consisted of the infectious construct cloned into the plasmid vector pAlter-1. In pC-35STMVR it had been recloned into the plasmid vector pcDNA II. Both these constructs were used for the manual inoculation of plants, infection of protoplasts and microprojectile bombardment experiments. The third construct pB-35STMV contained the 35S promoter driven clone of TMV, but did not have the ribozyme sequence at the 3' end of the viral genome. It was built in the binary vector pBin 19 and was therefore suitable for *Agrobacterium*-mediated plant transformation.

The manual inoculation of leaves was considered to be the simplest method for delivering constructs into the plant cell. The technique had been widely reported for investigating the infectivity of 35S-viral cDNA constructs built in other laboratories (see Section 1.4.3). The successful manual inoculation of *N. benthamiana* and *C. amaranticolor* with p35STMVR had been communicated to us by Dr. R. S. Nelson. However, attempts to manually inoculate *N. tabacum* cv. Xanthi plants had not been successful. We were able to confirm the ability of both, p35STMVR and pC-35STMVR, to infect *N. benthamiana*. Repeated attempts to induce a TMV infection by inoculating the constructs on to *N. tabacum* SR1, *N. tabacum* Samsun *NN*, *L. esculentum* GCR 26 (+/+) and *L. esculentum* GCR 237 (*Tm-1/Tm-1*) plants failed. The ability of p35STMVR to establish a TMV infection in both *N. benthamiana* and *C. amaranticolor* suggested that the 35S promoter maintained the ability to direct transcription of viral RNA. Furthermore the RNA was able to induce a TMV infection and did not appear to be compromised by the cloning procedure. This argument was supported by the fact that sap from the infected *N. benthamiana* and *C. amaranticolour* was highly infectious when manually

inoculated on to *N. tabacum* SR1 plants.

As described in Section 3.2.1 care had been taken to reduce the presence of non-viral 5' and 3' nucleotides following the transcription of p35STMVR (discussed in Section 1.4 and reviewed by Boyer and Haenni, 1994). The 35S promoter was directly fused to the cDNA clone of TMV-U1 in order to ensure that transcription started with the first nucleotide if the TMV genome. Likewise, most reported 35S-viral cDNA clones, used for manual inoculation experiments, have been carefully constructed to avoid the transcription of unwanted 5' nucleotides (MacFarlane *et al.*, 1992; Weber *et al.*, 1992; Maiss *et al.*, 1992; Dessens and Lomonossoff, 1993; Neeleman *et al.*, 1993; Ding *et al.*, 1995; Gal-On *et al.*, 1995). When designing these infectious clones care was also taken to reduce the number of 3' non-viral nucleotides transcribed (Section 1.4.3). It was presumed that the ribozyme was correctly functioning in p35STMVR and pC-35STMVR to reduce the number of 3' non-viral nucleotides. Inserting the ribozyme in the reverse orientation resulted in infectivity being decreased to ~7 % (Section 3.2.3; Dagless *et al.*, 1997). The results of microprojectile bombardment experiments (presented in Section 3.2.5, Figure 3.14 and 3.15) also suggested that the ribozyme was correctly functioning. *N. tabacum* SR1 leaf samples were bombarded with circular p35STMVR and pC-35STMVR. TMV coat protein and RNA was easily detected in detached leaf tissue at 4 days post bombardment. This construct should have undergone post-transcriptional ribozyme cleavage of RNA in order to limit 3' non-viral nucleotides. TMV coat protein and RNA was also detected at 4 days post bombardment with restricted pC-35STMVR. Restriction enzyme cleavage prior to bombardment should have ensured that 3' non-viral nucleotides were limited irrespective of whether the ribozyme was functioning. Ribozymes can be designed to cleave RNA at specific sites, leaving only a few non-viral nucleotides at the 3' end of transcripts. The advantage of ribozymes over a polyadenylation signal has been demonstrated. Turpen *et al.* (1993) used the method of agroinfection (described by Grimsley *et al.*, 1986) to inoculate *N. tabacum* Xanthi nc plants with a 35S-cDNA clone of TMV-U1. An ~50 % increase in infectivity was achieved when a 3' polyadenylation signal was replaced by a ribozyme sequence.

The procedure that was used for manual inoculation has been widely reported and does not appear to be at fault. Takahashi (1956) studied the optimum conditions required for the manual inoculation of TMV, two suggestions were made to increase infectivity. The first was that carborundum acted as a more effective abrasive if it was dusted on to leaves prior to inoculation instead of being mixed with the inoculum. This was observed by the inoculation protocol described in Section 2.10.1. The second suggestion was that TMV particles resuspended in phosphate buffer pH 8 as opposed to

water encouraged infection. It was presumed that this aided uncoating of the 5' end of the viral genome (Section 1.6.5; Wilson, 1984). It was therefore deemed unnecessary for the inoculation of plasmid DNA. The *in vitro* transcription of 1 μg of TMV cDNA by the T7 promoter has been shown to produce enough transcript for the efficient infection of one *N. tabacum* plant (Holt and Beachy, 1991). A similar amount of 35S-viral cDNA has been shown to be infectious to plants by direct manual inoculation (MacFarlane *et al.*, 1992; Neeleman *et al.*, 1993). However, results presented in Section 3.2.3 indicate that 10 μg of either p35STMVR or pC-35STMVR is required to ensure the reliable infection of *N. benthamiana.* This is consistent with other reports. 35S-cDNA clones of CPMV RNAs 1 and 2 were used to manually inoculate *Vigna unquiculata* plants (Dessens and Lomonossoff, 1993). Infections were observed providing the inoculum was made up of 10 μg of each clone. Likewise cDNA clones of CMV RNAs 1, 2 and 3 have been fused to 35S promoters (Ding *et al.*, 1995). A mixture containing 5 μg of each clone (15 μg of DNA in total) has been shown to be required for the reliable infection of the host plants by manual inoculation. Constructs also had to be excised from the plasmid vector prior to inoculation. Results reported in Section 3.2.3 show that increasing the amount of circular pC-35STMVR used for manual inoculation from 10 μg to 30 μg did not improve infectivity on *N. tabacum* plants. Using a 35S-TMV-L cDNA construct Weber *et al.* (1992) increased the amount of inoculum to 100 μg. The construct still failed to infect some plants known to host TMV including *L. esculentum* and *N. tabacum* Samsun *NN*. Virus was recovered from *N. tabacum* Samsun *nn* plants following inoculation with 100 μg of the 35S-TMV-L cDNA clone. However, this required a 4 week incubation period and two systemic passages.

Tests designed to determine the infectivity of 35S-viral cDNA constructs by manual inoculation have been carried out using a range of plants. This makes it difficult to compare results. Where possible results have been compared in order to establish whether some host plants are more susceptible to infection via manual inoculation. In agreement with our results Maiss *et al.* (1992) reported the successful infection of both *C. amaranticolor* and *N. benthamiana* using a 35S-cDNA clone of PPV. Furthermore, MacFarlane *et al.* (1992) reported that a 35S-cDNA clone of PEBV could infect *N. benthamiana* following the manual inoculation of leaves. Weber *et al.* (1992) reported that manual inoculation of a 35S-cDNA clone of TMV-L failed to induce a TMV infection in *L. esculentum* and *N. tabacum* Samsun *NN* plants. This is in agreement with the results presented in Section 3.2.3. Both Neeleman *et al.* (1993) and Ding *et al.* (1995) recommend that 35S-viral cDNA constructs should be released from their plasmid vectors prior to manual inoculation. Excision had previously been reported to aid the inoculation of cloned DNA viruses including CaMV (Howell *et al.*,

1980; Lebeurier *et al.*, 1980). Following excision Neeleman *et al.* (1993) reported the successful inoculation of *N. tabacum* Samsun *NN* plants with a 35S-cDNA clone of AlMV. Based on this advice the 35S-TMV cDNA insert of pC-35STMVR was excised from the pcDNA II vector. Following attempts to inoculate *N. tabacum* plants with the excised DNA insert two infections were observed (Section 3.2.3). One resulted in a single lesion appearing a *N. tabacum* Samsun *NN* plant (Figure 3.8). While it was likely that the lesion was induced by pC-35STMVR contamination from another source could not be ruled out. The second infection developed very slowly. As a result the *N. tabacum* SR1 plant was flowering by the time symptoms were visible. Like the observed single point of infection on the *N. tabacum* Samsun *NN* plant, this slow systemic spread of TMV may have been the result of initially infecting only one or very few cells. Taking into account all the attempts to infect *N. tabacum* plants with either p35STMVR or pC-35STMVR the two observed infections were equivalent to an ~4 % rate of infection. If only the attempts to inoculate *N. tabacum* plants with the excised 35S-viral cDNA insert are considered the rate of infection is closer to 10 %.

The study of transient gene expression in protoplasts offers the advantage of being able to synchronously inoculate large numbers of protoplasts. Levels of infection have been reported to be as great as 80-90 % (Takebe, 1975; Motoyoshi and Oshima, 1976). As a result the procedure has proved useful for the study of plant viruses. For example, TMV has been shown to multiply in protoplasts prepared from host cells expressing either the *Tm-2, Tm-2^2 or N* resistance genes (Sections 1.9.3 and 1.9.4). These observations helped us understand the way in which the genes conferred resistance in intact plants. In order to aid uptake of negatively charged virus particles they were pre-incubated with polycations, such as poly-L-ornithine, prior to the inoculation of protoplasts (Motoyoshi *et al.*, 1974). Motoyoshi *et al.* (1974) also suggested that sedimentation and resuspension of protoplasts immediately prior to inoculation may help disrupt the plasma membrane. As a result this was thought to aid the passive uptake of particles into the cytoplasm. As transcription occurs in the nucleus DNA constructs need to be transported across both the plasma membrane and the nuclear membrane. A number of techniques are available which facilitate uptake of such constructs, resulting in gene expression. The method described in Section 2.11.1 involves mixing DNA with a high concentration of PEG. It is thought that this long chain polymer may reduce charge repulsion effects between cellular membranes and DNA molecules. It has also been suggested that PEG may stimulate endocytosis (Draper *et al.*, 1986), a process known to actively transport molecules across the plasma membrane. The successful delivery of p35STMVR, presumably into the nucleus of *N. tabacum* SR1 leaf mesophyl cell protoplasts has been reported in Section 3.2 4. As a result infectious TMV particles were produced. It has been reported that

luciferase activity can be detected within 5-8 hours following the inoculation of protoplasts with 35S-LUC DNA constructs (Turner *et al.*, 1994). Furthermore TMV-coded proteins have been detected in protoplasts within 7 hours of inoculation with TMV RNA (Siegel *et al.*, 1978; Watanabe *et al.*, 1984a). It should therefore have been possible to detect TMV coat protein by 24 hours post inoculation with p35STMVR or pC-35STMVR following the careful preparation and inoculation of protoplasts.

Microprojectile bombardment has proven to be a valuable method for delivering both DNA and RNA into cells. The technique has been used for plant transformation, transient gene expression and also the delivery of viruses into cells. Early investigations involved bombarding tungsten particles coated in TMV RNA into *Allium cepa* cells (Klein *et al.*, 1987). Since then the method has successfully been used to overcome problems associated with the delivery of cloned DNA viruses into host cells. For example, Garzon-Tiznado *et al.* (1993) report the use of microprojectile bombardment for infecting intact *Capsicum annuum* plants with the geminivirus pepper huasteco virus (PHV). Later the particle bombardment technique was used to infect 65-88 % of *Theobroma cacao* seeds with cacao swollen shoot virus (CSSV) (Hagen *et al.*, 1994). CSSV is a mealybug transmitted badnavirus and is difficult to purify in quantities suitable for mechanical inoculation. The results presented in Section 3.2.5 demonstrate the use of microprojectile bombardment for the delivery of constructs p35STMVR and pC-35STMVR into *N. benthamiana*, *N. tabacum* SR1 and *L. esculentum* GCR 26 (+/+) plants. As a result TMV coat protein, RNA and particles were detected and highly infectious sap was produced. The normal host range of TMV appeared to be observed. p35STMVR failed to induce a TMV infection following bombardment into leaf tissue taken from non-host *P. sativum* plants. Furthermore, viral multiplication was significantly reduced when p35STMVR was bombarded into tissue from resistant *L. esculentum* GCR 237 (*Tm-1/Tm-1*) plants. Our results agree with a recent report where microprojectile bombardment was used to deliver a 35S-cDNA clone of ZYMV into *Cucurbitacae* seedlings (Gal-On *et al.*, 1995). The microprojectile bombardment procedure increased infection of the 35S-ZYMV cDNA clone from 19 % by manual inoculation to 100 %. It was observed that symptoms appeared much quicker following microprojectile bombardment, compared with manual inoculation with an equal dose of the 35S-ZYMV cDNA. This highlights the use of microprojectile bombardment for the efficient delivery of such constructs.

Detached leaf tissue from *N. tabacum* SR1 plants was independently bombarded with circular p35STMVR and pC-35STMVR as well as pC-35STMVR following either excision or linearisation. This led to TMV coat protein and RNA being

detected by 4 days post bombardment (Figures 3.14 and 3.15). The design of the experiment did not allow the accurate comparison of infectivity between constructs. It has been noted symptoms appeared quicker on *C. annuum* plants following microprojectile bombardment with circular plasmid containing cloned PHV as opposed to plasmid which had been restricted to excise the insert prior to bombardment (Garzon-Tiznado *et al.*, 1993). It was suggested that this may be due to the degradation of linear DNA. This could occur following delivery into plants and/or may be due to the toxic effect of tungsten microprojectiles. The advantages gained by linearising 35S-viral cDNA clones prior to manual inoculation (Neeleman *et al.*, 1993; Ding *et al.*, 1995) must outweigh any disadvantages induced as a result of increased degradation. By investigating particle bombardment using a 35S-GUS construct bound to gold particles it was shown that only 3 % of cultured *N. tabacum* cells contained a gold particle (in most cases this was a single gold particle). Only 7.5 % of these cell contained a gold particle in the nucleus (Yamashita *et al.*, 1991). The frequency of bombarding directly into the nucleus was calculated to be 2.2×10^{-3}. The authors identified a strong correlation >90 % between cells that expressed GUS and those that contained a gold particle in the cell nucleus.

Agrobacterium strain LBA4404 harbours a Ti plasmid which contains a viable *vir* gene but lacks the T-DNA region and 25 bp border sequences required for gene transfer. The *vir* gene is able to act, *in trans*, in order to mobilise the T-DNA region from the binary vector pBin 19 and transfer it into the plant cell. Section 3.2.6 describes the successful transformation of *N. tabacum* SR1 plants using the construct pB-35STMV. Eight plants were regenerated and PCR analysis confirmed all except U3 appeared to contain the stably integrated 35S-TMV cDNA construct in their genomes (Figures 3.17 and 3.18). The construct was able to produce highly infectious transcripts despite having no ribozyme or transcription termination signals to reduce the number of 3' non-viral nucleotides. Evidence suggests that once infection occurred 3' non-viral nucleotides would have been quickly lost from viral progeny (see Section 1.4.2; Boyer and Haenni, 1994). A 35S-cDNA clone of TMV-L was used to transform *N. tabacum* Samsun explants (Yamaya *et al.*, 1988). The regenerated plants demonstrated typical mosaic symptoms and sap from the plants induced lesions when inoculated on to *N. tabacum* Samsun *NN* plants. It is interesting that the transgenic line U plants as well as the plants regenerated by Yamaya *et al.* (1988) developed with mosaic symptoms. Murakishi and Carlson (1976) reported that it was possible to regenerate virus-free plants from the dark green areas of TMV infected tissue (Section 1.3). Furthermore virus-free plantlets demonstrated a transient state of resistance to TMV. The appearance of mosaic symptoms on the transgenic plants suggested that the resistance remained active even when TMV RNA should have been constitutively

expressed in all plant cells. It was attempted to separately sample leaf tissue from light and dark green areas of the regenerated plant U8. The level of TMV coat protein and RNA in the sample taken from dark green areas was lower than in the sample taken from light green areas (Figures 3.19 and 3.20). It was not possible to determine whether the presence of TMV coat protein and RNA in the dark green tissue sample was due to contamination from the heavily infected light green areas. The number of regenerated line U plants appeared to be very low. In particular the co-cultivation of ~60 explants in the second experiment gave rise to only one transgenic plant. This may be explained by the infectious nature of the disease coded by the 35S-TMV cDNA construct. Following cell division, young infected cells would have been required to support TMV replication along side their own development. This could account for increased cell abnormalities and death, leading to reduced numbers of regenerated plants.

TMV infections were detected in the regenerated *N. tabacum* SR1 plant U3 (which was not shown to be transgenic) and also in the centre of *N. tabacum* SR1 leaf discs co-cultivated with *Agrobacterium* containing pB-35STMV (Figure 3.16). This suggests that it may be possible to use *Agrobacterium* to inoculate plants with pB-35STMV without integrating the construct into all plant cells. Agroinfection was first described by Grimsley *et al.* (1986), as a means of infecting host plants with viruses which were not amenable to manual inoculation (reviewed by Grimsley, 1990). The technique has been used in order to infect dicotyledonous plants (Gardener *et al.*, 1986; Grimsley, *et al.*, 1986; Turpen *et al.*, 1993) as well as monocotyledonous plants which are not normally considered to be hosts of *Agrobacterium* (Grimsley *et al.*, 1988). Once cloned, plasmid containing viral clones can be easily transferred from *E. coli* and propagated within *Agrobacterium* cultures, prior to inoculation. Grimsley (1986) originally used agroinfection to infect plants with cloned CaMV. For the observed infections to have occurred the viral DNA must have escaped from the T-DNA region. Turpen *et al.* (1993) described the use of agroinfection to successfully infect *N. tabacum* Xanthi and *N. tabacum* Xanthi nc plants using a 35S-cDNA clone of TMV-U1. An infection was induced following the inoculation of leaves or injection of culture in to the stem or petiole. Care should of course be taken to avoid inoculating germ cells. As suitable methods (described in Section 3.2) were available for the infection of plants using p35STMVR and related constructs formal agroinfection experiments were not attempted.

Gene amplification and expression in eucaryotic cells requires that certain proteins and substrates are targeted into the nucleus by specific nuclear transport signals. This occurs via active transport through pores in the nuclear membrane. The

membrane remains intact except during cell division. Although machinery is in place for the one way transport of RNA out of the nucleus there is normally no requirement for the transport of either RNA or DNA into the nucleus. However, there are examples of DNA crossing into the cell nucleus. This is exploited by the use of *Agrobacterium* for plant transformation and agroinfection, and by the use of protoplasts for investigating gene expression. In addition, there are reports describing the successful manual inoculation of plants with cloned DNA and RNA viruses (Sections 1.4.3; 3.2.3). There are also examples of DNA moving out of the cell nucleus. Some viruses replicate within the host cell nucleus. It is essential for DNA to escape from the nucleus so that neighbouring cells can become infected. Studies of bean dwarf mosaic *geminivirus* (BDMV) have revealed two viral coded movement proteins. One facilitates movement of the virus from cell-to-cell and the other appears to assists the movement of DNA out of the cell nucleus (Noueiry *et al.*, 1994). It is unknown why p35STMVR and pC-35STMVR fail to infect *N. tabacum* and *L. esculentum* plants following manual inoculation, yet are highly infectious when inoculated on to *N. benthamiana* and *C. amaranticolor*. TMV is thought to replicate in the cytoplasm (Section 1.6.1; Satio *et al.*, 1987b; Hills *et al.*, 1987). It can therefore be hypothesised that the mechanical damage caused by manual inoculation may be sufficient for the delivery of virus particles and likewise DNA constructs into the cytoplasm. The difficulty may then arise due to constructs being incapable of crossing the nuclear membrane of some plants. Alternatively constructs may be degraded before transport can occur. It has been shown that DNA templates inoculated on to plants are totally degraded by 3 days post inoculation (van Emmelo *et al.*, 1987). The use of high speed microprojectiles may account for the delivery of DNA into the nucleus without damaging the cell. Gene expression following microprojectile bombardment appears to be dependant on DNA constructs being delivered directly into the nucleus (Yamashita *et al.*, 1991). The mechanisms by which foreign DNA reaches the nucleus via the other modes of delivery described in Section 3.2 are not fully understood.

In summary, the described protoplast, microprojectile bombardment and plant transformation experiments show that p35STMVR, pC-35STMVR and pB-35STMV are viable. When successfully delivered into host cells they are capable of being transcribed to produce viral RNA. Following expression of the TMV replicase proteins the RNA can replicate and produce functional subgenomic RNAs. The ability to manually inoculate all host plants with the constructs would have been favourable. However, the described successful methods of infection (resulting in the production of infectious sap) were sufficient to allow the investigations reported in future Chapters.

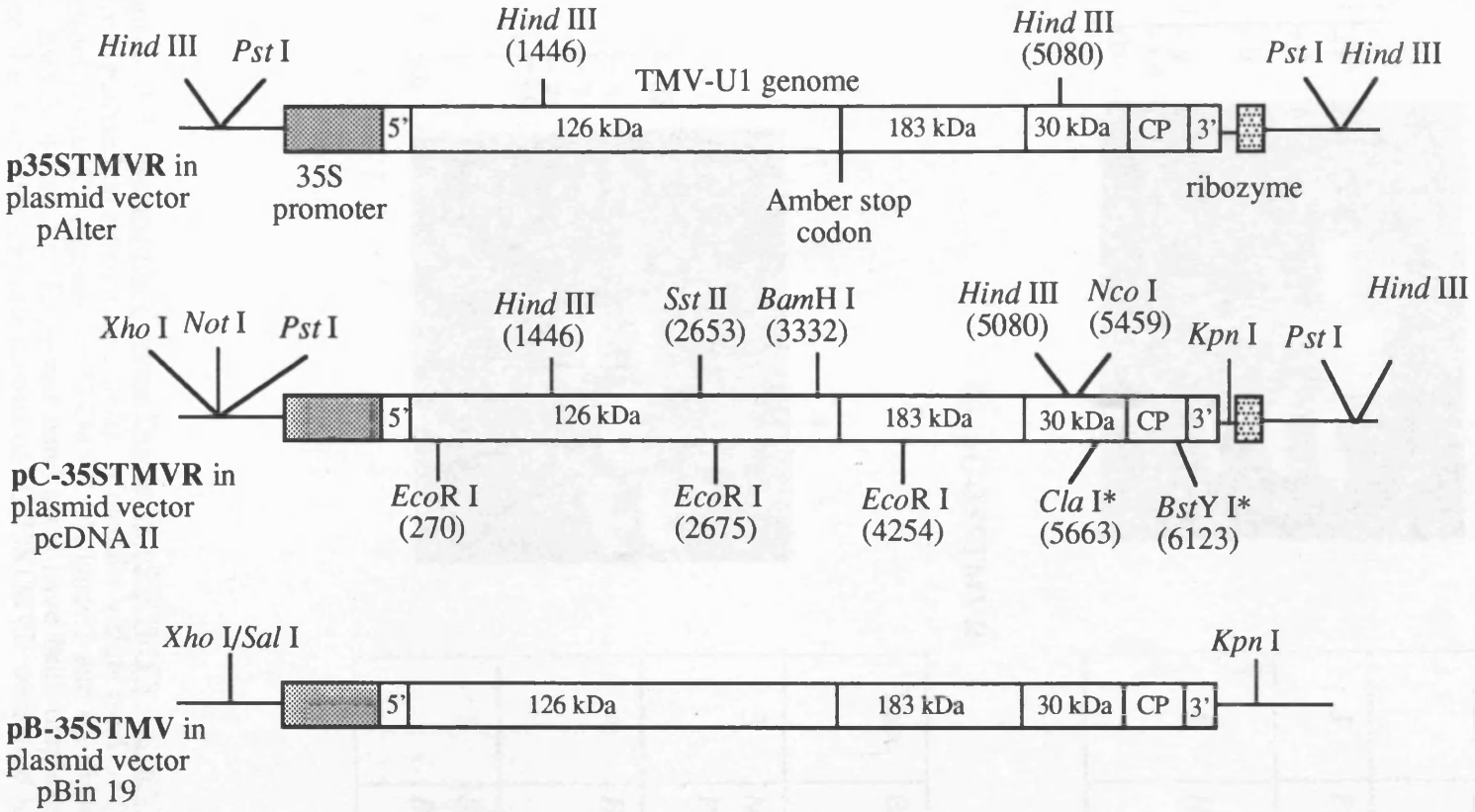
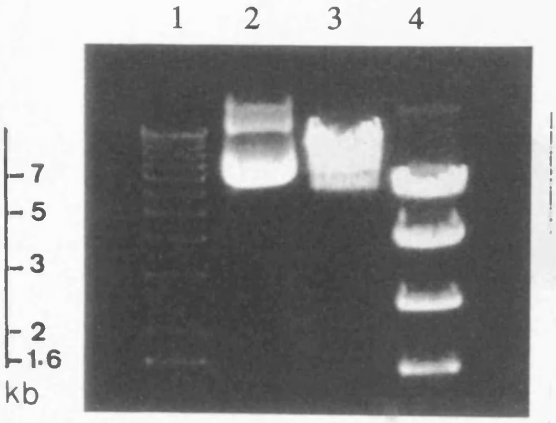


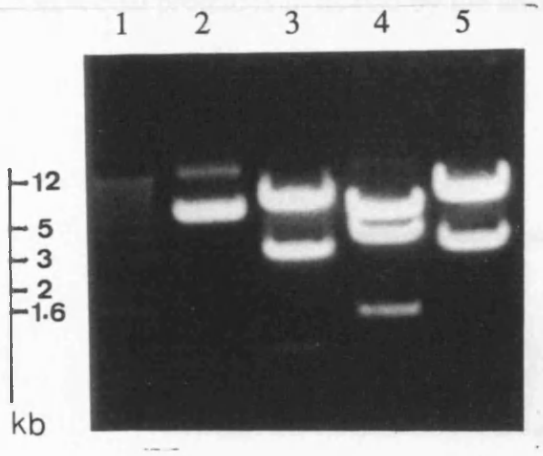
Figure 3.1 Diagrams of p35STMVR, pC-35STMVR and pB-35STMV. Restriction enzyme sites used for the cloning and diagnosis described in section 3.2.2 have been indicated. Sites within the TMV genome, used for cloning and diagnosis throughout this thesis, have been shown on the diagram of pC-35STMVR. Numbers refer to nucleotides of the TMV-U1 genome and correspond to the nucleotide immediatly 5' from the point of cleavage. 17.5 kDa coat protein ORF (CP).
* indicates that sites are not unique.

A) p35STMVR



Lane	Enzyme	Expected fragment size
3	*Pst* I	7.1 kb
		5.7 kb
4	*Hind* III	5.7 kb
		3.6 kb
		2.2 kb
		1.3 kb

B) pC-35STMVR



Lane	Enzyme	Expected fragment size
3	*Nco* I	6.2 kb
	Pst I	2.9 kb
		0.9 kb
4	*Hind* III	1.3 kb
		3.6 kb
		5.1 kb
5	*Sst* II	6.2 kb
	*Bam*H I	3.1 kb
		0.7 kb

Figure 3.2 Restriction Enzyme Digests of p35STMVR and pC-35STMVR. Digests were visualised on agarose gels (left). Molecular weight markers (lanes 1), undigested plasmid (lanes 2), digested p35STMVR (A, lanes 3 and 4), digested pC-35STMVR (B, lanes 3, 4 and 5). Expected band sizes have been displayed in tables (right). Note, the restriction enzyme digests of pC-35STMVR were used to orientate the insert within the vector.

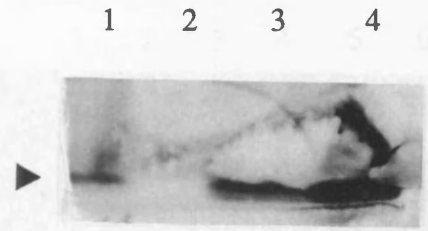


Figure 3.3 Western Immunoblot to Detect TMV Coat Protein in *N. benthamiana* Following Manual Inoculation with p35STMVR and pC-35STMVR. Protein samples were prepared from uninoculated leaf tissue, probed with antibody raised to the TMV-U1 coat protein and visualised using an alkaline phosphatase conjugated secondary antibody. Purified coat protein (lane 1), sample taken from an uninoculated plant (lane 2). Approximately equal concentrations of protein, prepared from plants at 5 days post manual inoculation with 10 μg of p35STMVR (lane 3); pC-35STMVR (lane 4). 17.5 kDa coat protein is indicated by the arrow.

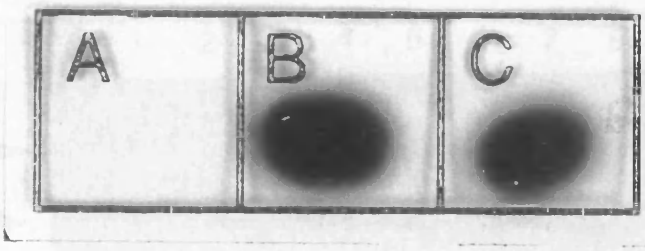


Figure 3.4 Dot Blot to Detect TMV RNA in *N. benthamiana* Following Manual Inoculation with p35STMVR and pC-35STMVR. Extracts prepared from uninoculated leaf tissue were probed with an ~3.6 kb *Hind* III fragment isolated from pC-35STMVR and exposed to X-ray film for 24 hours. Extract prepared from an uninoculated plant (A). Approximately equal concentrations of extract prepared from plants at 5 days post manual inoculation with 10 μg of p35STMVR (B); pC-35STMVR (C).

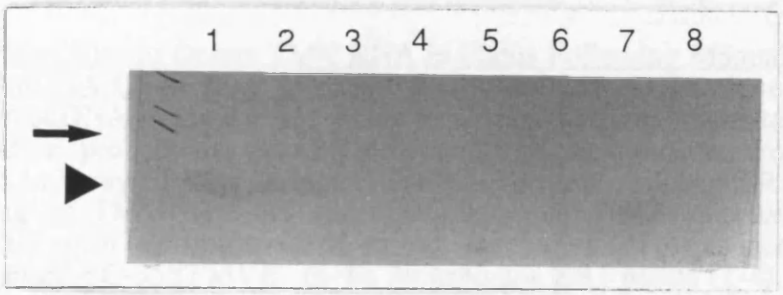


Figure 3.5 Western Immunoblot to Detect TMV Coat Protein in Plants at 12 Days Post Manual Inoculation with Either TMV Particles or p35STMVR. Approximately equal concentrations of protein were extracted from uninoculated leaves, probed with antibody raised to the TMV-U1 coat protein and visualised using an alkaline phosphatase conjugated secondary antibody. Protein extracted from plants inoculated with ~10 µg of purified TMV-U1 particles (lanes 1-3); *N. tabacum* SR1 (lane 1); *L. esculentum* GCR 26 (+/+) (lane 2); *L. esculentum* GCR 237 (*Tm-1/Tm-1*) (lane 3). Protein extracted from plants inoculated with 10 µg of p35STMVR (lanes 4-6); *N. tabacum* SR1 (lane 4); *L. esculentum* GCR 26 (+/+) (lane 5); *L. esculentum* GCR 237 (*Tm-1/Tm-1*) (lane 6). Purified TMV coat protein (lane 7), extract from uninoculated *N. tabacum* SR1 (lane 8). 17.5 kDa coat protein (▶), 24 kDa protein marker (⟶).

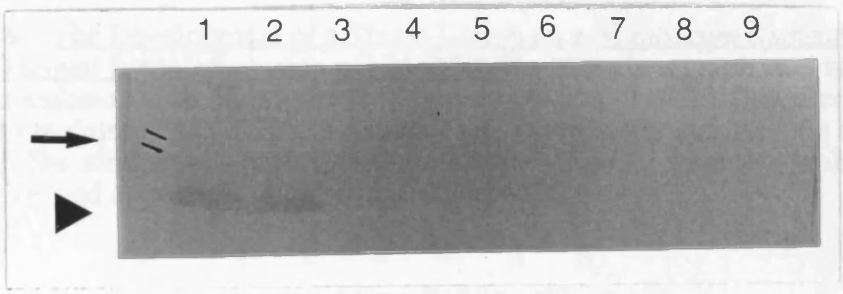


Figure 3.6 Western Immunoblot to Detect TMV Coat Protein in Plants at 25 Days Post Manual Inoculation with Either TMV Particles or p35STMVR. Approximately equal concentrations of protein were extracted from uninoculated leaves, probed with antibody raised to the TMV-U1 coat protein and visualised using an alkaline phosphatase conjugated secondary antibody. Protein extracted from plants inoculated with ~10 µg of TMV particles (lanes 1-4); *L. esculentum* GCR 26 (+/+) (lanes 1 and 2); *L. esculentum* GCR 237 (*Tm-1/Tm-1*) (lanes 3 and 4). Extracts from plants inoculated with 10 µg of p35STMVR (lanes 5-9); *L. esculentum* GCR 26 (+/+) (5 and 6); *L. esculentum* GCR 237 (*Tm-1/Tm-1*) (lanes 7 and 8); *N. tabacum* SR1 (lane 9). 17.5 kDa coat protein (▶), 24 kDa protein marker (⟶).

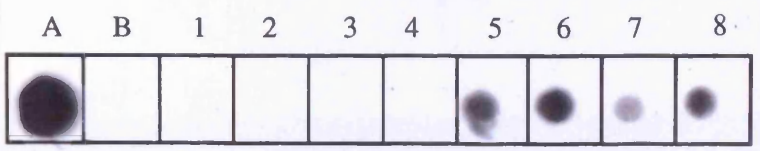


Figure 3.7 Dot Blot to Detect TMV RNA in Plants Following Manual Inoculation with the Excised cDNA Clone from pC-35STMVR. The 35S-cDNA clone of TMV-U1 was excised from pcDNA II using *Pst* I. Samples were taken from inoculated leaves at 5 days post inoculation, probed with an ~3.6 kb *Hind* III fragment isolated from pC-35STMVR and exposed to X-ray film for 24 hours. Sample from a *N. tabacum* SR1 plant inoculated with ~10 µg of TMV particles (A); an uninoculated *N. tabacum* SR1 plant (B). Approximately equal concentrations of extract from individual plants inoculated with 10 µg of *Pst* I digested pC-35STMVR (1-8); *N. tabacum* SR1 plants (1-4); *N. benthamiana* plants (5-8).

Figure 3.8 The Development of a Single Lesion on a *N. tabacum* Samsun *NN* Leaf Following Manual Inoculation with pC-35STMVR. The photograph was taken at 12 days post inoculation with 30 µg of *Pst* I digested pC-35STMVR. The insert shows a dot blot used to detect TMV RNA in tissue at and immediately surrounding the site of the lesion. The sample was probed with an ~3.6 kb *Hind* III fragment isolated from pC-35STMVR and exposed to X-ray film for 24 hours.

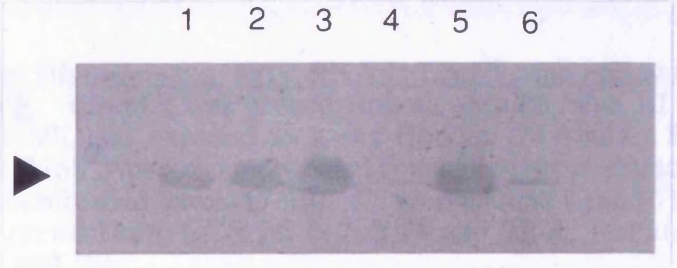


Figure 3.9 Western Immunoblot Showing TMV Coat Protein in *N. tabacum* SR1 Tissue Following Infection by p35STMVR. Purified TMV-U1 coat protein (lane 1), protein sampled from tissue manually inoculated with ~10 µg of TMV particles (lane 2). Using p35STMVR protein samples were prepared at 24 hours following the inoculation of protoplasts (lane 4); microprojectile bombardment into detached leaf tissue (lane 6). Samples prepared from uninoculated leaves, taken at 7 days post manual inoculation of plants, with the protoplast extract (lane 3); sap from the bombarded leaf tissue (lane 5). 17.5 kDa coat protein is indicated by the arrow.

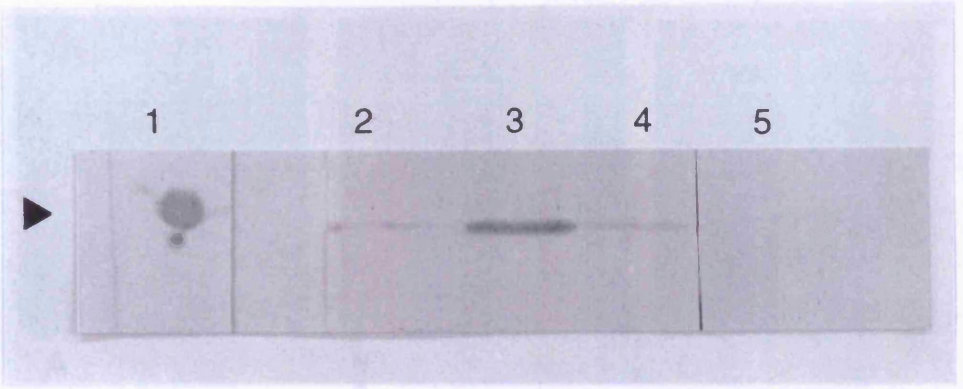


Figure 3.10 Western Immunoblot Showing TMV Coat Protein in Leaf Tissue Following Bombardment with p35STMVR. Protein was probed with antibody raised to the TMV-U1 coat protein and visualised using an alkaline phosphatase conjugated secondary antibody. Purified TMV coat protein (lane 1). Approximately equal concentrations of protein prepared from bombarded leaf tissue (lanes 2-5); *N. benthamiana* (lane 2); *N. tabacum* SR1 (lane 3); *L. esculentum* GCR 26 (+/+) (lane 4); *L. esculentum* GCR 237 (*Tm-1/Tm-1*) (lane 5). 17.5 kDa coat protein is indicated by the arrow.

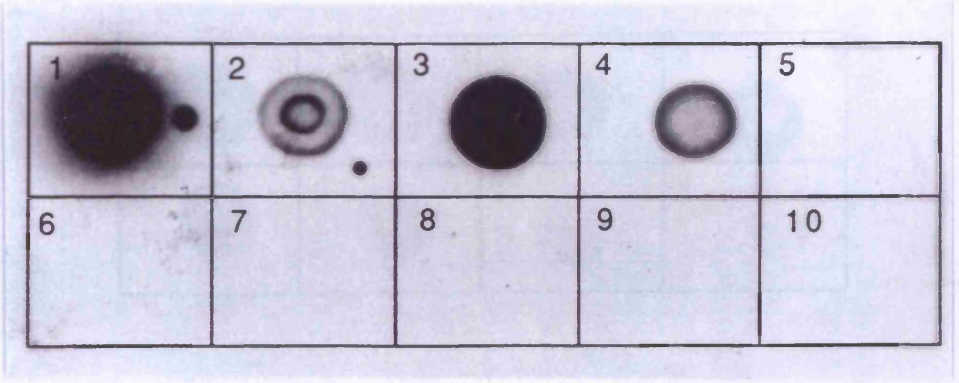


Figure 3.11 Dot Blot Showing TMV RNA in Leaf Tissue Following Bombardment with p35STMVR. The blot was probed with an ~3.6 kb *Hind* III fragment isolated from pC-35STMVR and exposed to X-ray film for 24 hours. Purified TMV-U1 particles (1), dH_20 (6). Approximately equal concentrations of extract from bombarded leaves (2-5); unbombarded leaves (7-10); *N. benthamiana* (2 and 7); *N. tabacum* SR1 (3 and 8); *L. esculentum* GCR 26 (+/+) (4 and 9); *L. esculentum* GCR 237 (*Tm-1/Tm-1*) (5 and 10).

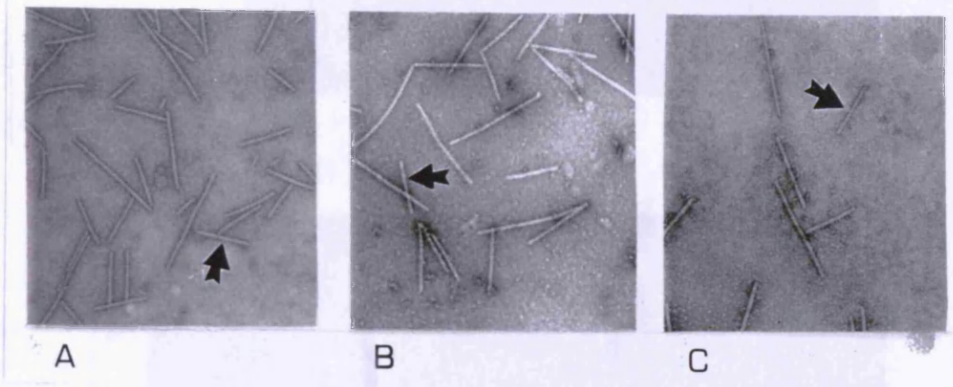


Figure 3.12 Transmission Electron Micrographs Showing TMV Particles in Leaf Tissue Following Bombardment with p35STMVR. The magnification was 2.5×10^4. Full length TMV particles (300 nm) have been indicated by the arrows. Approximately equal concentrations of extract from *N. benthamiana* (panel A); *N. tabacum* SR1 (panel B); *L. esculentum* GCR 26 (+/+) (panel C).

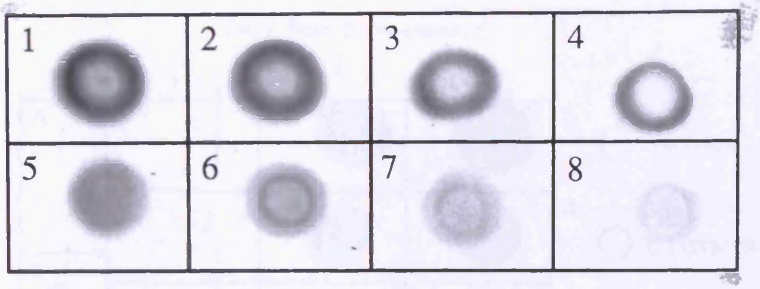


Figure 3.13 Dot Blot Showing TMV RNA in *N. tabacum* SR1 Plants Following Manual Inoculation with Sap from Bombarded Tissue. Leaf tissue was bombarded with p35STMVR and incubated for 5 days, sap was then used to inoculate *N. tabacum* SR1 plants which were grown for a further 7 days prior to analysis. The blot was probed with an ~3.6 kb *Hind* III fragment isolated from pC-35STMVR and exposed to X-ray film for 24 hours. Approximately equal concentrations of extract (1-4), 1:10 dilution of extracts (5-8). Plants were inoculated with the following p35STMVR bombarded tissue; *N. benthamiana* (1 and 5) *N. tabacum* SR1 (2 and 6); *L. esculentum* GCR 26 (+/+) (3 and 7); *L. esculentum* GCR 237 (*Tm-1/Tm-1*) (4 and 8).

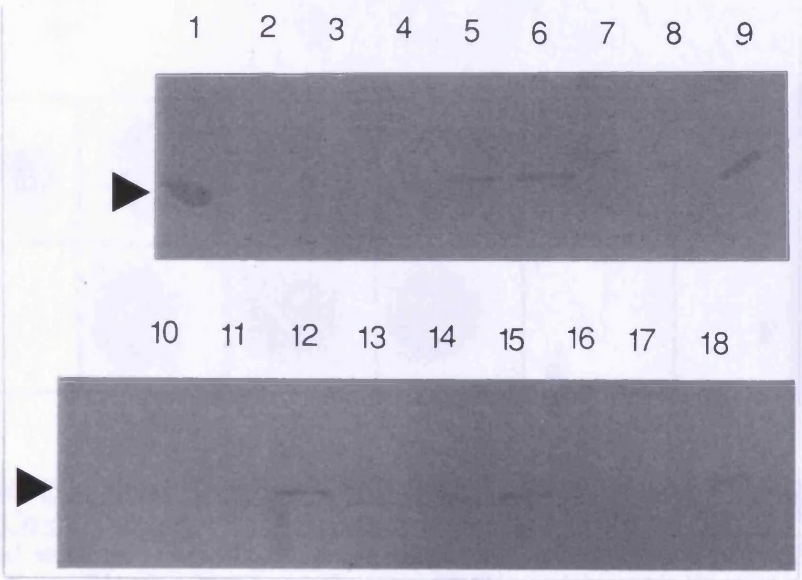


Figure 3.14 Western Immunoblot to Detect TMV Coat Protein in *N. tabacum* SR1 Tissue Following Bombardment with Circular and Digested p35STMVR. Protein was probed with antibody raised to TMV-U1 coat protein and visualised using an alkaline phosphatase conjugated secondary antibody. Purified TMV coat protein (lane 1); blank/unloaded (lane 2); protein prepared from an unbombarded leaf (lane 3). Approximately equal concentrations of protein prepared at two (lanes 4, 7, 10, 13 and 16), four (lanes 5, 8, 11, 14 and 17) and six (lanes 6, 9, 12, 15 and 18) days post bombardment with, circular p35STMVR (lanes 4-6); circular pC-35STMVR (lanes 7-9); *Pst* I digested pC-35STMVR (lanes 10-12); *Kpn* I digested pC-35STMVR (13-15); *Xho* I digested pC-35STMVR, (16-18). 17.5 kDa coat protein is indicated by the arrows.

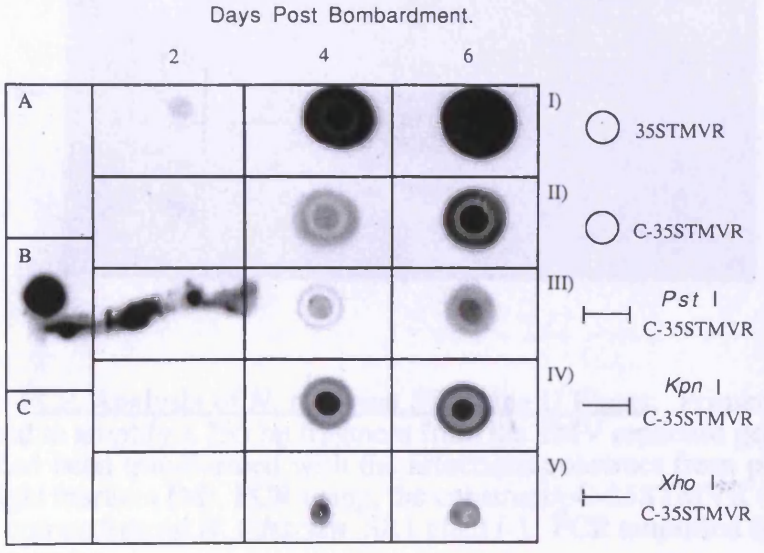


Figure 3.15 Dot Blot Showing TMV RNA in *N. tabacum* SR1 Tissue Following Bombardment with Circular and Digested p35STMVR. The blot was probed with an ~3.6 kb *Hind* III fragment isolated from pC-35STMVR and exposed to X-ray film for 24 hours. dH_2O (A), purified TMV particles (B) and extracts from an unbombarded leaf (C). Extracts were prepared from approximately equal amounts of tissue at two, four and six days post bombardment with circular p35STMVR, circular pC-35STMVR, *Pst* I digested pC-35STMVR, *Kpn* I digested pC-35STMVR and *Xho* I digested pC-35STMVR.

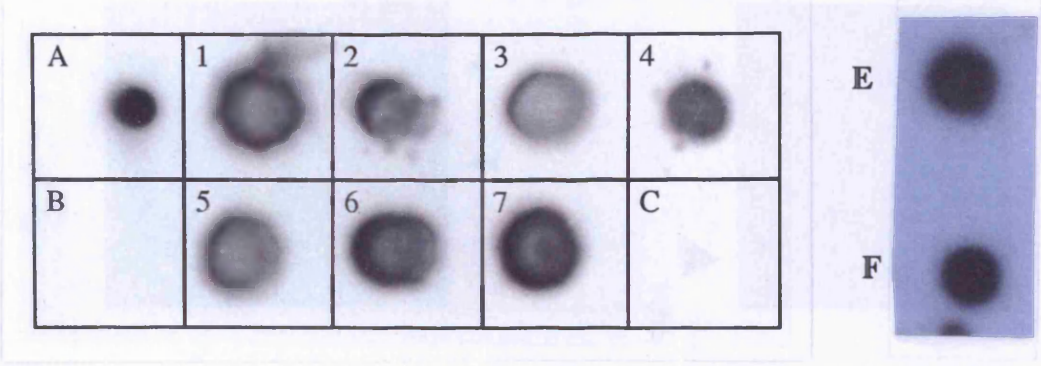


Figure 3.16 Dot Blots Showing TMV RNA in Explants and Regenerated Plants Following Co-cultivation with *Agrobacterium* Harbouring pB-35STMV. The blots were probed with an ~3.6 kb *Hind* III fragment isolated from pC-35STMVR and exposed to X-ray film for 24 hours. Purified TMV particles (A); Extract prepared from a healthy, untransformed *N. tabacum* SR1 leaf (B); dH_20 (C). Approximately equal concentrations of extract were prepared from *N. tabacum* SR1 plant lines U1-U7 (panel number refers to plant line). Samples taken from the centre of 2 explants at >6 weeks post co-cultivation (E and F).

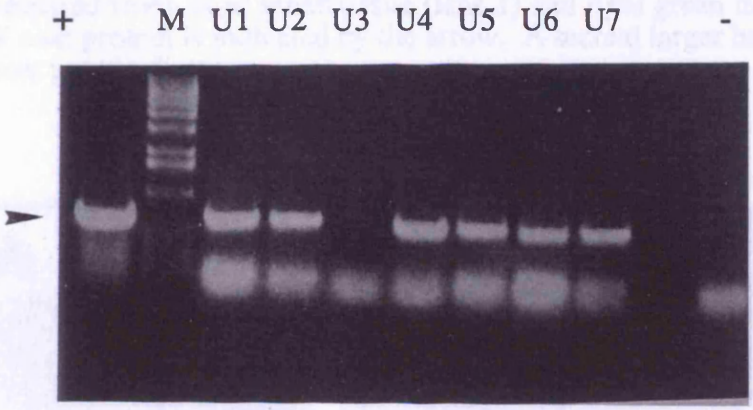


Figure 3.17 PCR Analysis of *N. tabacum* SR1 Line U Plants. Primers 5'REP and 3'REP were used to amplify a 735 bp fragment from the TMV replicase genes of line U plants which had been transformed with the infectious construct from pB-35STMV. Molecular weight markers (M). PCR using, the construct pC-35STMVR (+); genomic DNA from an untransformed *N. tabacum* SR1 plant (-). PCR amplified fragments are indicated by the arrow.

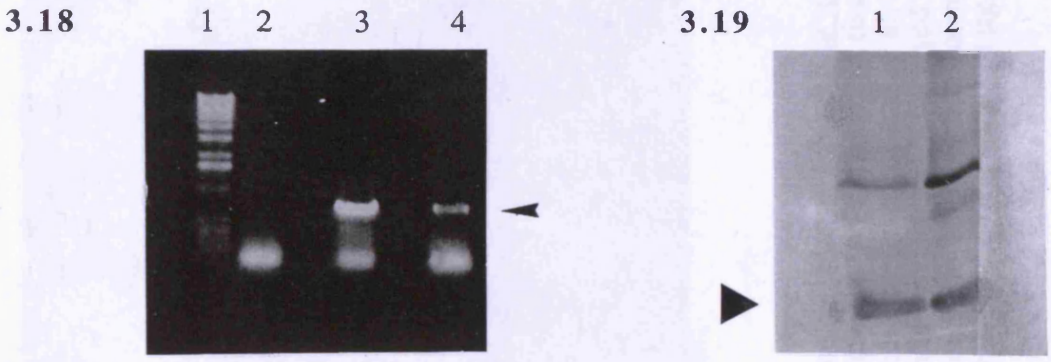


Figure 3.18 PCR Analysis of the *N. tabacum* SR1 Plant U8. Primers 5'REP and 3'REP were used to amplify a 735 bp fragment from the TMV replicase genes of plant U8 which had been transformed with the infectious construct from pB-35STMV. Molecular weight markers (lane 1). PCR using genomic DNA prepared from an untransformed *N. tabacum* SR1 plant (lane 2), the construct pC-35STMVR (lane 3); genomic DNA prepared from plant U8 (lane 4). Amplified fragments are indicated by the arrow.

Figure 3.19 Western Immunoblot Showing TMV Coat Protein in Plant U8. Protein was probed with antibody raised to TMV-U1 coat protein and visualised using an alkaline phosphatase conjugated secondary antibody. Approximately equal amounts of protein were prepared from dark green tissue (lane 1) and light green tissue (lane 2). 17.5 kDa TMV coat protein is indicated by the arrow. A second larger band may have been due to a coat protein dimer.

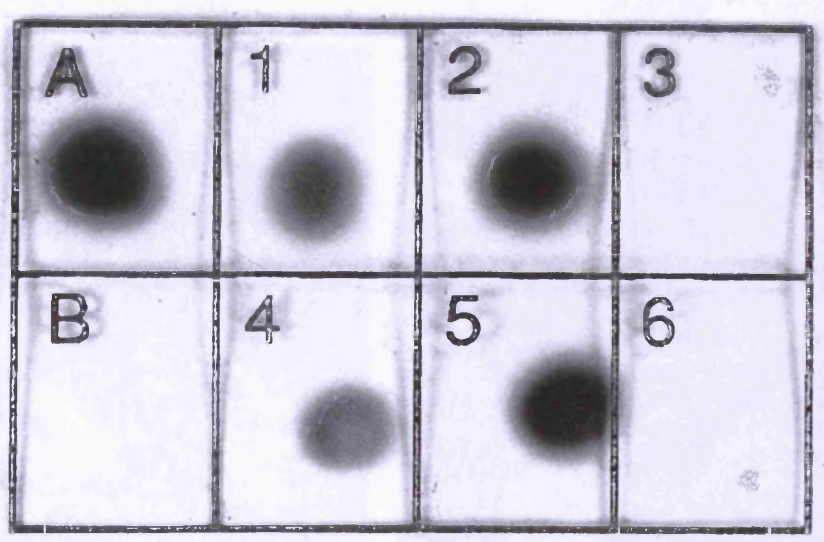


Figure 3.20 Dot Blot Showing TMV RNA in Plant U8. The blot was probed with an ~3.6 kb *Hind* III fragment isolated from pC-35STMVR and exposed to X-ray film for 24 hours. Purified TMV particles (A); dH_2O (B). Approximately equal concentrations of extract (1-3), 1:10 dilution of extract (4-6). Extract from dark green tissue isolated from plant U8 (1 and 4); light green tissue isolated from plant U8 (2 and 5); a healthy untransformed *N. tabacum* SR1 plant (3 and 6).

A)

B)

C)

Figure 3.21 Symptoms Displayed by Line U Plants Expressing the Infectious Construct from pB-35STMV. Plant U1 (A) remained in tissue culture until photographed. Plant U8 (B) was transferred to soil to allow further growth and development. A comparison of symptoms (C) displayed by a leaf from plant U8 (left) and a systemically infected untransformed *N. tabacum* SR1 leaf, following manual inoculation of a plant with ~10 µg of purified TMV particles (right).

CHAPTER 4

THE DESIGN AND ANALYSIS OF TWO REPLICATION-MARKER CONSTRUCTS AND A CONTROL REPLICASE-MARKER CONSTRUCT

4.1 INTRODUCTION

The development of virus-based expression vectors for the production of foreign protein is potentially very important (discussed in Section 1.8). The ability of TMV to multiply to high titres within plant cells makes it a good candidate on which to base investigations. To date much of the work has centred around either replacing or inserting sequences into the coat protein ORF. As a result, both small polypeptides (Takamatsu *et al.*, 1990a; Hamamoto *et al.*, 1993; Turpen *et al.*, 1995) and larger reporter genes (up to 1 kb) have been successfully expressed (Dawson *et al.*, 1989; Donson *et al.*, 1991; Chen *et al.*, 1996). It is thought that the use of viral expression vectors will be commercially important, for example, for the production of vaccines. However such vectors may also be useful for the study of viruses and the way in which they replicate. The role of the TMV encoded 126 kDa and 183 kDa proteins in replication and the production of subgenomic mRNAs has been discussed (see Section 1.5.1). Ishikawa *et al.* (1986) demonstrated that production of both of the proteins was required for normal virus replication (Section 1.7.1). The TMV encoded 30 kDa protein and 17.5 kDa proteins are not required for replication (Meshi *et al.*, 1987; Takamatsu *et al.*, 1987; Dawson *et al.*, 1988). Until recently, attempts to isolate an active TMV replication complex had failed (Section 1.6.2; Osman and Buck, 1996). As a result, studies of the way in which TMV replicates were hindered. Instead information was provided by the study of other viruses with similar viral replicase genes. These include BMV (Miller *et al.*, 1985; Miller *et al.*, 1986) and Sindbis virus (Hahn *et al.* 1989). The *Tm-1* gene is partially dominant and confers resistance to TMV in *L. esculentum* (Section 1.9.2). It is thought that the gene acts on the viral replicase genes to reduce multiplication by as much as 95 % (Fraser and Loughlin, 1980). By analysing resistance breaking strains, Meshi *et al.* (1988) observed that two nucleotide substitutions in the coding region for the replicase proteins conferred the ability to overcome the *Tm-1* gene. In particular a substitution at position 3006 of the TMV-L genome, resulting in Glutamic acid replacing Glutamine, was important. It was suggested that the substitutions reduced the local net charge and in doing so altered the way in which the replicase proteins may interact with the *Tm-1* gene (Meshi *et al.*, 1988).

The initial aim of this project was to build a TMV-based expression vector which would facilitate isolation of the *Tm-1* gene via insertional mutagenesis (Section 1.10). A marker gene was designed to be driven by the TMV-U1 coat protein subgenomic promoter. Given that the *Tm-1* gene conferred resistance by inhibiting viral replication (Section 1.9.2), it was hoped that expression of the marker gene would be allowed if the *Tm-1* gene was no longer functioning. It was decided that the

viral constructs should be expressed in every cell of a plant, following stable integration into the genome. This removed the requirement for either cell-to-cell or long distance movement. As a result it was thought that replacement of the TMV 17.5 kDa coat protein gene (Section 1.7.3) and interruptions to the 30 kDa cell-to-cell movement protein gene (Section 1.7.2) would be tolerated. This Chapter described the construction and analysis of the two "replication-marker" constructs. A third construct, the "replicase-marker" construct, has also been described. It lacked a 3' untranslated region (UTR) and as a result transcribed RNA should have been incapable of replication and the expression of subgenomic RNAs (Section 1.5.1; 1.6.4). The purpose of this construct was to demonstrate whether expression of the marker gene was dependent on viral replication.

4.2 RESULTS

4.2.1 Choice of the Marker Gene

Before choosing a suitable marker/reporter gene a number of points were considered.

i) The project required that the constructs were transformed into *L. esculentum* plants, however, initial analysis was to be carried out using *N. tabacum* plants. It was therefore important that high levels of the marker protein were tolerated by both plant species.

ii) It was necessary to establish whether endogenous levels of the marker protein, or other plant proteins would interfere with expression and/or activity assays.

iii) If required, it was essential that sensitive activity assays should be as simple as possible.

Use of the firefly (*Photinus pyralis*) luciferase gene (LUC) as a highly sensitive marker had previously been demonstrated (Ow *et al.*, 1986). In the presence of luciferin, ATP, magnesium and oxygen the luciferase protein catalyses the formation of an excited enzyme-bound product (DeLuca and McElroy, 1978). Upon decomposition light is emitted which can be measured by a simple assay using a luminometer (see Section 2.13.3). Alternatively intact plants soaked in luciferin can be exposed to X-ray film. Unfortunately the luciferase enzyme is not very stable. In addition, care must be taken to ensure suitable reaction conditions for the assays. The β-Glucuronidase gene (GUS) isolated from *E. coli* is a hydrolase enzyme which catalyses the cleavage of β-glucuronides (Jefferson *et al.*, 1986; Jefferson *et al.*, 1987). A number of substrates are available for the study of GUS activity. Quantitative fluorimetric analysis can be carried out using MUG (Section 2.13.4.1). Alternatively the use X-GLUC allows qualitative histochemical analysis (Section 2.13.4.2). The protein is stable in

plants, and can tolerate storage at -20°C. The *R* genes regulate expression of the red/purple anthocyanin pigment in *Zea mays* (Ludwig *et al.*, 1990). In order to achieve this the genes are driven by different regulatory promoters. An *R* gene such as *Lc* can be an effective visual marker in plants species which are capable of expressing anthocyanin. Such plants include *N. tabacum* (Lloyd *et al.*, 1992) and *L. esculentum* (personal observation). However, it is important to distinguish between endogenous anthocyanin production and the pigment produced following expression of a foreign marker gene. Recently green fluorescent proteins from *Aequorea victoria* (described by Cody *et al.*, 1993) have received much attention. When excited GFPs emit a strong green fluorescence. Activity can be monitored in a number of ways including visualisation under UV light or quantitative spectroscopy. Techniques are not yet as sensitive as those used to detect luciferase or GUS activity (Baulcombe *et al.*, 1995).

It was decided that quantitative data concerning the activity of replication-marker constructs was essential for preliminary analysis. The sensitivity and simplicity of luciferase assays therefore promoted use of the luciferase gene in such constructs. Once a suitable construct had been developed replacement of the gene by a visual marker, such as the *R* gene *Lc* or a GFP gene, was to be considered.

4.2.2 Generation of the Constructs

4.2.2.1 The Marker Cassettes

Three different marker cassettes were built pS4-L1, pSLU and pSLLUTR, a diagrammatic representation of the cloning strategy is shown in Figures 4.1-4.4. The cDNA clone 4-25 (Dawson *et al.*, 1986) was used to provide the TMV sequences required for construction of the cassettes. It consisted of the 3' end of the TMV-U1 genome downstream of the *Hind* III site at nucleotide 5080 and had been inserted into the plasmid vector pUC18. Using the sequence published by Goelet *et al.*, (1982) oligonucleotide primers were designed to amplify the coat protein subgenomic promoter. The Primer 5P-1, 5' GGTCTAGAGTTGATGAGTTCATG 3', matched nucleotides 5482-5496 of the TMV-U1 genome. The 5' end of this primer contained an *Xba* I restriction enzyme site which was to be introduced into the PCR product. The primer 5P-2N, 5' CGGATCCATGGTTTAATACGAATCCG 3', was complementary to nucleotides 5696-5710 of the TMV-U1 genome, with the exception of TMV nucleotide 5705 where T replaced A. The 5' end of the primer 5P-2N was designed to introduce *Nco* I and *Bam*H I restriction enzyme sites into the PCR product. The combined use of 5P-1 and 5P-2N resulted in the amplification of 229 nucleotides from the TMV-U1 coat protein subgenomic promoter of clone 4-25.

Both the PCR generated fragment and the plasmid pBS SK$^-$ were digested using *Xba* I and *Bam*H I. The vector and insert were added to a ligation reaction and correctly cloned products were identified. One, designated pSGP4 (Figure 4.1), was selected and sequenced using the M13 Reverse and M13 -20 Forward primers (primer sites were present at opposite ends of the pBS SK$^-$ multiple cloning site). The sequence data revealed that the PCR amplification had introduced 3 base substitutions. At nucleotide 5487 of the TMV-U1 genome C replaced T, at nucleotide 5585 G replaced A and at nucleotide 5705 (described earlier) G replaced A. Two of these substitutions coincided with primer binding sites and therefore suggested that the mistakes occurred during synthesis of the primers. The ~1.7 kb luciferase gene was excised from the construct pRTV2-LUC (Turner *et al.*, 1994) using *Nco* I and *Bam*H I. Following purification it was cloned into *Nco* I, *Bam*H I digested pSGP4 resulting in the construct pS4-L1 (Figure 4.1). The construct containing the TMV coat protein subgenomic promoter fused to the luciferase gene was the first marker cassette to be generated. It was used to build the replicase-marker construct which was designed to be incapable of replication.

The primers 3P-1 and 3P-2 were designed using the sequence of TMV-L (Ohno *et al.*, 1984). It was intended to use them to amplify the 3' untranslated region from either TMV-L or TMV-U1 cDNA. 3P-1, 5' CGGATCCTCTGCACCTGCAT CTT 3', almost matched nucleotides 6172-6189 of the TMV-U1 genome. There were three differences. Based on the TMV-U1 sequence (Goelet *et al.*, 1982), at nucleotides 6178 C replaced G, at nucleotide 6179 A replaced T and at nucleotide 6186 T replaced A. The 5' end of the primer contained a *Bam*H I restriction enzyme site. The primer 3P-2, 5' CGGATCCTGGGCCCCAACCGGGGGT 3', was complementary to nucleotides 6378-6395 of the TMV-U1 genome, with the exception of one base substitution. At nucleotide 6387 A replaced T. Again a *Bam*H I restriction enzyme site was introduced at the 5' end of the primer. The primers 3P-1 and 3P-2 were used in order to amplify a fragment from cDNA clone 4-25. This fragment consisted of the final 224 nucleotides from the 3' end of the TMV-U1 genome and was flanked by *Bam*H I restriction enzyme sites. Using these sites the PCR product was digested and cloned into *Bam*H I digested, dephosphorylated pBS SK$^-$. A correctly cloned plasmid, designated p3'UA (Figure 4.2), was selected and sequenced using the M13 Reverse and M13 -20 Forward primers. The sequence data revealed that the 5' end of the TMV 3' untranslated region was closest to the M13 Reverse primer site. No nucleotide substitutions were identified other than those introduced by the primers (described above). The 3' untranslated region was excised from p3'UA using the *Bam*H I restriction sites. It was then ligated into *Bam*H I digested, dephosphorylated pS4-L1. PCR analysis, using the M13 primers, was used in order to identify plasmids

containing the 3' untranslated region in the correct orientation. The construct pSLU was selected (Figure 4.2). Essentially it consisted of the TMV-U1 genome downstream of nucleotide 5482 with the coat protein gene replaced by the luciferase gene. This marker cassette was used to build one of the replication-marker constructs.

The 3' untranslated region was cloned via a second method. The primer 3P-1N, 5' CGGATCCCCACGACTGCCGAAACG 3', matched nucleotides 6017-6035 of the TMV-U1 genome. It contained a *Bam*H I restriction enzyme site at the 5' end. The primer was used in conjunction with the M13 Reverse primer in order to amplify a fragment from the cDNA clone 4-25. The PCR product should have contained the final 379 nucleotides from the 3' end of the TMV-U1 genome. The amplified fragment (LUTR) was digested using *Bam*H I and *Pst* I. Following purification it was ligated into similarly digested pS4-L1, the resulting construct was designated pSLLUTR (Figure 4.3). Sequencing was carried out using the M13 -20 Forward primer. The data obtained extended into the TMV-U1 genome, from the 3' end, as far as nucleotide 6041. It showed that nucleotides 6041-6390 of the TMV-U1 genome remained unchanged. However, the remaining 5 TMV nucleotides (6391-6395) were missing, instead there was a *Pst* I restriction enzyme site followed by sequence of unidentified origin. The cDNA clone 4-25, in the plasmid vector pUC18, was digested using *Pst* I and *Pst* I, *Hind* III. The result indicated that the plasmid no longer contained a recognisable *Pst* I restriction enzyme site. Purified plasmid thought to contain the cDNA clone 4-25 was sequenced using the M13 Reverse primer. This should have been situated downstream of the 3' end of the TMV genome, however, no TMV sequence was identified. A series of mutations/contaminations appeared to have been introduced into the plasmid stock, how or when these occurred remains unclear. pSLLUTR contained the marker cassette which was used to generate a second replication-marker construct. However, it was accepted that due to the missing terminal 5 nucleotides from the TMV 3' untranslated region the region may have been incapable of directing viral replication (Section 1.6.4).

All three marker cassettes were excised from p BS SK$^-$ using *Not* I and *Pst* I. They were then ligated into the plasmid vector pcDNA II which had been restricted using the same enzymes. The new marker cassettes containing beneficial restriction enzyme sites were designated pCS4-L1, pCSLU and pCSLLUTR (see Figure 4.4).

4.2.2.2 The Replicase Cassette

The construct p35STMV in the plasmid vector pAlter-1 has been discussed in Chapter 3. It contained the CaMV 35S promoter directly fused to the 5' end of a full

length cDNA clone of TMV-U1 (see Figure 3.1). As a result it was a suitable source of the 126 kDa and 183 kDa replicase genes which should have been efficiently expressed *in planta* by the 35S promoter and 5' untranslated leader sequence (Section 1.6.3). The multiple cloning site from pSL301 was excised using *Nco* I and *Kpn* I and ligated into similarly digested p35STMVR. As a result useful restriction enzyme sites replaced the 3' portion of the TMV-U1 genome, downstream from the *Nco* I site at nucleotide 5459. A diagrammatic representation of the replication cassette designated pU-mcs is shown in Figure 4.5.

4.2.2.3 Joining the Cassettes

The marker cassettes, pCS4-L1, pCSLU and pCSLLUTR, were excised from pcDNA II using *Xho* I and *Kpn* I and ligated into *Sal* I, *Kpn* I digested pU-mcs (*Xho* I and *Sal* I are compatible but can not be redigested). The completed replicase-marker construct was designated pU-S4-L1, while the replication-marker constructs were designate pU-SLU and pU-SLLUTR (See Figure 4.6). It was necessary for the completed constructs to be transferred into binary vectors prior to plant transformation. In order to gain suitable restriction enzyme sites they were first transferred from pAlter to pcDNA II. The constructs were excised from pAlter-1 using flanking *Pst* I sites. Following purification they were ligated into *Pst* I digested, dephosphorylated pcDNA II. Restriction enzyme digests, Southern blot analysis and PCR analysis were used to confirm the identity and orientation of the constructs (Figures 4.7; 4.8; 4.9). The result showed that in each case the 35S promoter was closest to the M13 -20 Forward primer site. The intermediate constructs designated pCU-S4-L1, pCU-SLU and pCU-SLLUTR were used for the microprojectile bombardment experiments described in Section 4.2.4. The replicase-marker construct and two replication-marker constructs were finally excised from pcDNA II using *Xho* I and *Kpn* I and ligated into *Sal* I, *Kpn* I digested pBin 19. The new binary constructs were designated pBU-S4-L1, pBU-SLU and pBU-SLLUTR (Figure 4.7).

4.2.3 *In Vitro* Analysis of the Luciferase Gene

Before analysing the completed replicase-marker and replication-marker constructs the viability of the luciferase gene used to prepare the constructs was tested. pCSLU and pCSLLUTR both contained the T7 promoter site upstream of the luciferase gene (Figure 4.4). This allowed transcription to be conducted *in vitro* according to the method described in Section 2.9.1. The constructs were linearised downstream of the 3' untranslated region using *Kpn* I prior to transcription. The resulting RNAs were used for *in vitro* translation reactions (Section 2.9.2). The sequence upstream of the

luciferase start codon consist of part of the pBS SK$^-$ multiple cloning site and the TMV-U1 coat protein subgenomic promoter. It was thought that this sequence would be tolerated by ribosomes, although it was likely to prevent optimum expression of the luciferase gene. 5 µl of the *in vitro* translation products were assayed for luciferase activity (Section 2.13.3). The readings obtained were 120 660 LU and 107 500 LU for pCSLU and pCSLLUTR respectively. Both were ~100x higher than the background reading obtained, following the analysis of a blank tube.

4.2.4 Analysis of the Constructs Using Microprojectile Bombardment

Transient assay experiments were carried out to determine the ability of the replicase-marker and replication-marker constructs to function in the cells of plants known to host TMV. Both the inoculation of protoplasts and microprojectile bombardment experiments were carried out according to methods described in Section 2.11. Three attempts were made to inoculate *N. tabacum* SR1 protoplasts with either ~10 µg of one of the marker constructs or ~10 µg of a control 35S-LUC construct pRTS2-LUC (Turner *et al.*, 1994). However, high levels of luciferase expression were not observed. The low expression following inoculations using pRTS2-LUC indicated that the procedure had repeatedly failed. The results of the protoplast experiments have not been presented and no further inoculations were attempted. Delivery of the constructs into plant tissue via microprojectile bombardment was more successful. The majority of experiments involved bombarding tissue with ~1.15 µg of one of the marker constructs pCU-S4-L1, pCU-SLU or pCU-SLLUTR and ~0.25 µg of a 35S-GUS internal control construct pRTL2-GUS (Restrepo *et al.*, 1990). Each marker construct was independently bombarded into 36 target tissue samples (~2.25 cm^2). 33 were prepared from *N. tabacum* leaf tissue and 3 were prepared from *N. benthamiana* leaf tissue. All recorded data has been presented in Tables 4.1-4.11 which relate to Experiments 4.1-4.11 respectively. The optimum bombardment conditions described in Section 2.11.2 were maintained in all experiments. However, some experimental conditions were altered, these have been described in the table legends. Information provided in previous legends has not been repeated. Three different types of DNA were used for the bombardments i) Circular plasmid DNA ii) Plasmid linearised downstream of the luciferase gene and TMV 3' untranslated region, using *Xho* I iii) The replicase-marker or replication-marker cassettes excised from the pcDNA II vector, using *Pst* I. Following digestion the DNA was phenol:chloroform extracted, precipitated using ethanol and resuspended in dH_2O (~1 µg µl^{-1}). The target tissue was incubated for between 1-6 days post bombardment prior to assaying for luciferase and GUS activity.

Results have been presented using the following terms i) Total luminescence (in light units) measured for 10 seconds following the addition of luciferin and ATP to 50 µl of extract (Section 2.13.3) ii) Luciferase activity, calculated by subtracting the background reading (measured for 1 second prior to the addition of luciferin and ATP) from total luminescence iii) GUS activity, representing fluorescent units per minute emitted when tissue extract was mixed with MUG (Section 2.13.4.1) iv) Relative luciferase activity, calculated by dividing the luciferase activity by the GUS activity. Successful bombardments were indicated by a GUS activity, measured at the same time as luciferase activity, of >20 FU min^{-1}. The bombardment procedure appeared to be successful in all experiments where internal GUS activities were calculated, with the exception of Experiment 4.4. It should also be noted that the GUS activities obtained following the co-bombardment of pRTL2-GUS with pCU-SLU in Experiment 4.3 and pCU-S4-L1 in Experiment 4.6 were also low (<20 FU min^{-1}). Although the internal control construct pRTL2-GUS was included in Experiments 4.9-4.11 GUS assays were not performed on the bombarded tissue samples. The luciferase activities observed, following the analysis of unbombarded leaf samples, indicate that endogenous luminescence (upon the addition of luciferin and ATP) in the region of 1000 LU may be expected (Table 4.1). The control 35S-LUC construct pRTS2-LUC (Turner *et al.*, 1994) was bombarded into *N. tabacum SR1* tissue as part of Experiment 4.2. The average luciferase activity following 3 bombardment was 3 933 540 LU.

The luciferase activities observed following the bombardment of either pCU-S4-L1, pCU-SLU or pCU-SLLUTR into *N. tabacum* SR1 tissue were low. The highest average luciferase activities obtained from Experiment 4.6, were <5x higher than the activities determined following the analysis of unbombarded leaves (Table 4.1). In addition they were ~1000x lower than the activities observed following the bombardment pRTS2-LUC into *N. tabacum* SR1 tissue (Table 4.2). In Experiment 4.6 the highest relative luciferase activity was obtained from tissue samples bombarded with pCU-S4-L1. RNA transcribed by this replicase-marker construct was designed to be incapable of replication. The next highest value was observed in tissue bombarded with pCU-SLLUTR. Although designed as replication-marker construct, a deletion at the 3' end of the TMV 3' untranslated region meant RNA transcribed by pCU-SLLUTR may have been incapable of replication. The only results showing a higher relative luciferase activity in tissue bombarded with the construct pCU-SLU are presented in Table 4.4. However, the low GUS activities obtained suggested that the experiment had not been very successful. The results presented in Table 4.3 also suggest that luciferase activity may have been highest in tissue bombarded with pCU-SLU. Unfortunately the background activities were not recorded for this experiment and as a result relative luciferase activities could not be accurately calculated.

No luciferase activities were detected as a result of Experiments 4.5, 4.7 and 4.9. Extremely low average luciferase activities (<300 LU) were detected following the bombardment of pCU-SLLUTR in Experiment 4.8. No luciferase activities were detected as a result of the other bombardments conducted as part of this Experiment. Microprojectile bombardment was used to deliver the constructs independently into three tissue samples prepared from *N. benthamiana* the results are presented in Table 4.10. Again the average luciferase activities remained low or zero. GUS assays were not performed on the bombarded tissue and so relative luciferase activities could not be calculated. Although it was difficult to compare results obtained from different experiments, no obvious differences in luciferase activities were detected.

4.2.5 The Generation of Transgenic Plants

The replicase-marker construct and two replication-marker constructs were designed to be integrated into the genome of *N. tabacum* SR1 plants. This was carried out according to the methods described in Section 2.12. The binary constructs pBU-S4-L1, pBU-SLU and pBU-SLLUTR were conjugated independently into *Agrobacterium* strain LBA4404. Each separate culture was then co-cultivated with 120 freshly prepared *N. tabacum* SR1 leaf disc explants. Shoots started to develop by 4 weeks post co-cultivation, these were transferred to powder rounds containing MSO medium to encourage root development. The development of kanamycin resistant roots indicated that the regenerated plants were transgenic. Genomic DNA was also analysed using PCR in order to confirm that the constructs had been incorporated into the plant genome. The primers 5'REP and 3'REP (Section 3.2.6) were used in order to amplify a 735 bp fragment from the TMV-U1 126 kDa and 183 kDa replicase protein ORFs.

Thirteen kanamycin resistant plants were regenerated following the co-cultivation of leafdisc explants with *Agrobacterium* harbouring the construct pBU-S4-L1. They were numbered and given the prefix "L". The PCR analysis of lines L3, L4, L5, L6, L7, L8, L10 L12 and L13 suggested that the replicase-marker cassette had been integrated into the plant genome of lines L3, L4, L6, L7, L8 and L12 (Figure 4.10). The plants regenerated following the co-cultivation of explants with *Agrobacterium* harbouring either pBU-SLU or pBU-SLLUTR were numbered and given the prefix "T" or "C" respectively. Genomic DNA prepared from a selection of the regenerated plants was analysed using PCR, the results have been presented in Figure 4.11. In some cases two independent genomic DNA preparations, from the same plant line, were analysed. PCR amplification from only one of these preparations was taken to indicate that the plant was transgenic. As some of the bands were faint

following agarose gel electrophoresis the results were confirmed by Southern blot analysis. DNA was probed with the ~3.6 kb *Hind* III fragment isolated from pC-35STMVR, nucleotides 1446-5080 of the TMV-U1 genome. The analysis revealed faint signals in samples prepared from untransformed *N. tabacum* SR1 tissue. However, the intensity of these signals was low, compared with the signals considered to be positive. Similar signals were also present in samples taken from plants not judged to be transgenic. Out of the 23 line T plants regenerated PCR analysis indicated that T1, T3, T4, T6, T12, T14, T15, T16 T17, T20 and T 22 contained the stable integrated replication marker construct from pBU-SLU. Plant lines T2, T7, T8, T9, T10, T11, and T 23 were not analysed. All 12 regenerated line C plants were analysed using PCR. The results indicated that the plant lines C1, C2, C3, C4, C5, C7, C8, C9 and C11 contained the stable integrated replication marker construct from pBU-SLLUTR.

While in tissue culture leaf samples were removed from the regenerated line L and line T plants and assayed for luciferase activity. The resulting luciferase activities were not significantly higher than the endogenous luminescence observed in control leaves (see Table 4.1). The highest luciferase activity of 6 110 LU was observed in plant T3. As the protein concentration of samples was not measured the results were incomplete and have not been presented. The T_0 plants were transferred to soil and allowed to flower, the seed was then collected.

4.2.6 Analysis of the T_1 Generation

4.2.6.1 Segregation Analysis

Seeds collected from the regenerated T_0 plants L6, L7, L8, L12, T 3, T6, T14, T17, T20, C2, C4, C5, C8, and C11 were sterilised and plated on MSO plates containing 100 μg ml kanamycin. The plates were incubated in a plant growth room for 4 weeks. The number of seeds which developed into healthy seedlings was recorded, these seedlings were considered to be kanamycin resistant. Bleached seedlings and those which failed to develop leaves other than the two cotyledons were not included. The Figures were compared with the number of seeds which germinated from each line (Table 4.12). The number of germinated seeds ranged from 43-107. The number of shoots resistant to kanamycin ranged from 62-94 %. Two of the seedlings were selected from each line, transferred to soil and designated either A or B. PCR analysis using primers 5'REP and 3'REP was performed on genomic DNA samples prepared the "A" plants. The result (not presented) indicated that all plant lines contained genes for the TMV-U1 replicase proteins integrated into their genomes.

4.2.6.2 Analysis of Luciferase Activity

Two samples ~2.25 cm^2 were removed from each plant and assayed to determine their luciferase activities. The protein concentration of each sample was also measured (according to the method described in Section 2.13.5). The results are presented in Tables 4.13, 4.14 and Figure 4.13. It was apparent that the plants were expressing higher than background levels of luciferase. With the exception of plants L6A, L8A, L12A, C4A, C11A, L6B, L8B and C8B the average luciferase activities (LU mg $protein^{-1}$) were >10x higher than the average activity of 795 LU determined for *N. tabacum* SR1 leaf samples (Table 4.1). This value was obtained following the analysis of two leaf samples ~2.25 cm^2. However, the protein concentration of the samples was not measured. The transgenic line L, T and C plants had been transferred to soil for at least 4 weeks and were undergoing a period of growth prior to flowering at the time when samples were taken. The second sample was removed from the plants for analysis seven days after removal of the first sample. It is interesting to note that the luciferase activities calculated following analysis of the first sample were higher than for the second sample in the majority of cases. For some reason the two values tended to be closer for the A plants than for the B plants. In particular, the calculated luciferase activity for the first sample removed from plant C11B was 89 979 LU mg $protein^{-1}$ compared with 16 069 LU mg $protein^{-1}$ for the second sample. The pattern of luciferase expression also varied between A and B plants (Figure 4.13). This is highlighted by plant line C11 where the average luciferase activity calculated for line A plants was 7 682 LU mg $protein^{-1}$ and 55 024 LU mg $protein^{-1}$ for line B plants. Some variation between A and B plants may be expected due to the way in which chromosomes carrying the transgenic inserts segregated in individual plants. It was thought that the line T plants may have been transformed with the most active replication-marker construct (from pBU-SLU). As a result these plants were expected to express the highest levels of luciferase. However, the highest average level of luciferase expression was observed in plant C11B which had been transformed replication-marker construct from pBU-SLLUTR. This construct carried a deletion in the TMV 3' untranslated region. Other plants with average luciferase activities above 30 000 LU mg $protein^{-1}$ (in order of decreasing activity) were L7B > C2A > L12B > T20A. All the data was compiled to determine the average luciferase activities for plants transformed with each type of construct. The activities were all fairly similar. The highest activity of 18 782 LU mg $protein^{-1}$ was determined for line C plants transformed with the replication-marker construct from pBU-SLLUTR. This was followed by a value of 16 154 LU mg $protein^{-1}$ for line T plants transformed with the replication-marker construct from pBU-SLU. The lowest activity of 14 444 LU mg $protein^{-1}$ was calculated for line L plants transformed with the

replicase-marker construct from pBU-S4-L1.

4.2.6.3 Inoculation of Plants with TMV

It was decided to determine whether the transgenic plants, expressing part of the TMV-U1 genome, were resistant to the virus. Samples, ~1 cm^2 were removed from each of the T_1 generation "A" plants and used for RNA dot blot analysis. Sap was probed with the ~3.6 kb *Hind* III fragment isolated from pC-35STMVR. TMV RNA remained undetected following exposure to X-ray film for 24 hours (result not presented). A suitable leaf was selected from each plant and inoculated with ~5 µg of TMV-U1 (Section 2.10.1). Samples were removed from upper uninoculated leaves at 10 days post inoculation. Sap samples were again probed with the ~3.6 kb *Hind* III fragment isolated from pC-35STMVR. The result following exposure to X-ray film for 24 hours has been presented in Figure 4.12. TMV RNA was detected in all the plants except L6A, T14A and C8A. Furthermore, the signals tended to be weaker in the line T plants analysed. These had been transformed with the replication-marker construct from pBU-SLU. The described experiment was only a preliminary investigation aimed at establishing whether the transgenic plants were resistant to TMV. Further experiments, with the inoculation of suitable controls, were required in order to provide more substantial results. Results from similar experiments aimed at investigating resistance to TMV in transgenic plants have been presented in Sections 6.2.3.3 and 7.2.4.3.

4.3 DISCUSSION

Section 4.2 describes the design, production and analysis of 3 constructs, pU-S4-L1, pU-SLU and pU-SLLUT. Each has been described in 3 plasmid vectors which suited individual experimental requirements. For convenience general references to the constructs do not always refer to a particular plasmid vector. Each construct was made up of an identical replication cassette pU-mcs (Figure 4.5) which was fused to separate marker cassettes (Figures 4.4-4.6). RNA transcribed by the replication-marker constructs, pU-SLU and pU-SLLUTR, was designed to mimic the way in which TMV replicates and produces subgenomic RNAs (Section 1.5.1). The aim was to allow replication-dependent expression of a luciferase marker gene. Such a construct would be a useful tool for monitoring levels of viral replication at the cellular level. The specific aim of this project was to develop a replication-marker construct which could be included in a strategy designed to isolate the *Tm-1* resistance gene, by transposon tagging (Section 1.10). The construct was to be used to determine whether the gene

was functioning and therefore inhibiting viral replication (Section 1.9.2). The replicase-marker construct pU-S4-L1 was designed as a control construct. It lacked the TMV 3' untranslated region and as a result transcribed RNA should have been incapable of replication (Section 1.6.4).

Repeated microprojectile bombardment experiments were carried out to determine whether the constructs were capable of expressing luciferase in plants known to be hosts of TMV. In total each construct was independently bombarded into 33 target tissue samples prepared from *N. tabacum* SR1 plants and 3 prepared from *N. benthamiana* plants. The results were disappointing, no luciferase activities significantly higher than background luminescence were detected (Tables 4.1-4.11). The samples were initially assayed at one day post bombardment. This incubation period is sufficient for the expression of high level of luciferase in tissue bombarded with pRTS2-LUC (Table 4.2). It was suggested that if the bombarded tissue was incubated for a longer period continued replication may result in increased luciferase expression. This is supported by the results presented in Section 3.2.5, which describe the microprojectile bombardment of p35STMVR and pC-35STMVR into *N. tabacum* SR1 tissue. The TMV coat protein was not easily detected until 4 days post bombardment (Figures 3.14 and 3.15). These results are supported by the observation that, synthesis of TMV coat protein reaches a maximum rate between 37 and 71 hours post inoculation of intact plants with TMV particles (Joshi *et al.*, 1983; Moser *et al.*, 1988). There were disadvantages associated with the continued incubation of bombarded leaf tissue. These arise due to the unstable nature of DNA, RNA and the luciferase protein. There is evidence to suggest that all DNA is degraded by 3 days post inoculation of plant tissue (van Emmelo *et al.*, 1987). It was suggested that luciferase expression may be improved if the constructs were either linearised or excised from their vectors prior to bombardment. It has been shown that excised 35S driven viral cDNA constructs can be more infectious than constructs contained within circular plasmids, following the manual inoculation of plants (Neeleman *et al.*, 1993; Ding *et al.*, 1995). However, results of microprojectile bombardment experiments suggest that linear DNA is less stable than circular plasmid DNA (Garzon-Tiznado *et al.*, 1993). The viral coat protein plays an essential role in stabilising genomic and subgenomic RNAs (Section 1.7.3). However, the replicase-marker and replication-marker constructs did not contain the coat protein ORF. Furthermore, when the constructs were co-bombarded with pC-35STMVR protection from the viral coat protein was still not expected. This was due to destruction of the OAS (Section 1.6.7), situated within the 30 kDa protein ORF of TMV-U1, during the cloning procedure (Figure 4.6).

Despite the disappointing microprojectile bombardment results, the replicase-maker and replication-marker constructs were integrated into the genomes of *N. tabacum* SR1 plants. It was thought that luciferase expression significantly higher than background luminescence would be detected if a replication-marker construct was present in every cell of a plant. PCR analysis of genomic DNA was used to confirm the transgenic nature of regenerated kanamycin resistant T_0 plants (Figure 4.10 and 4.11). While PCR provides a quick method for identifying the presence of transgenes, it should be noted that there are problems associated with the technique. The contamination of samples with foreign DNA can led to false positives. In addition, successful PCR amplification requires certain optimum reaction conditions. If these are not provided for example, due to impurities present in genomic DNA preparations false negative results can be observed. This could explain why following the preparation of two independent DNA samples from plants T1 and T4 only one contained suitable template for successful PCR amplification. Assays to determine levels of luciferase activity were performed on 14 selected T_1 generation plants. Segregation analysis suggested that plants L6 and T20 may have contained 2 copies of the transgenic insert, while the other lines contained only a single copy (Table 4.12; Croy, 1993). Although higher than background luminescence, the luciferase activities observed in the transgenic tissue remained low compared with the activities observed in tissue bombarded with pRTS2-LUC (Table 4.2). Furthermore, it was not possible to distinguish between line L, T and C plants (Figure 4.13). Out of the 3 marker cassettes generated pSLU was the only one to contain an authentic TMV-U1 untranslated region (except for one base substitution at the position equivalent to nucleotide 6387 of the TMV-U1 genome). As a result it was thought that RNA replication, leading to the production of subgenomic RNAs and expression of the luciferase gene, may only occur in plants transformed with pBU-SLU. It is possible that RNA transcribed by the construct pBU-SLLUTR may have been capable of replication despite lacking the 5 terminal TMV 3' untranslated nucleotides (Section 4.2.2.1). However, it is not clear why luciferase activity, above background luminescence, was observed in plants transformed the construct pBU-S4-L1. It is possible that the breakage or degradation of RNA transcribed by the 35S promoter allowed the eukaryotic ribosomes to access the luciferase gene. Alternatively, following translation of the viral replicase genes, reinitiation may have occurred upstream of the luciferase gene ORF.

From the results presented in Sections 4.2.4 and 4.2.6.2 it can be concluded that the replication-marker constructs were not capable of expressing luciferase in the manner intended. The design of the constructs was critically analysed in an attempt to understand why pU-SLU, in particular, failed to function. *In vitro* analysis of the

luciferase gene (Section 4.2.3) suggested that the cloned gene was not at fault. A number of authors have made reports describing the construction of similar TMV based constructs (Section 1.8). A selection of these were studied prior to designing the replication-marker constructs. Two of the reports described replacing the TMV coat protein gene with the foreign gene (Takamatsu *et al.*, 1987; Dawson *et al.*, 1988). As a result transcripts were incapable of efficient systemic movement (Section 1.7.3). An alternative approach was to add an additional ORF to those already coded by the TMV genome (Dawson *et al.*, 1989; Donson *et al.*, 1991). The generation of an infectious replication-marker construct was not necessary for the project as it was intended to integrate a suitable construct into the genome of plants. As a result we decided to replace the coat protein ORF with the luciferase gene. Experiments conducted by Meshi *et al.* (1987) were designed to investigate the way in which deleted cDNAs of TMV-L functioned. It was demonstrated that the deletion of nucleotides 4936-6187, which included almost the entire 30 kDa protein ORF as well as the coat protein ORF, did not effect the ability of transcribed RNA to replicate. The inability of replication-marker constructs to form virus-like particles and to move from cell-to-cell as well as systemically through out plants was considered to be an advantage. While the potential of virus based expression vectors is recognised there is concern over risks associated with the technology (Hull, 1990). The removal of unnecessary genes, which may assist the escape of vectors into the field, can be seen as an important objective.

No known fault could be assigned to the replication cassette. This consisted of the 35S promoter fused to the 5' portion of the TMV-U1 genome, as far as the *Nco* I site at nucleotide 5459. The 35S-cDNA fusion (described in Section 3.2.1) was carefully constructed in order to ensure that transcription began with the first nucleotide of the TMV-U1 genome. The exclusion of non-viral 5' nucleotides can be essential for the production of infectious transcripts (Section 1.4.2; Dawson *et al.*, 1986; Janda *et al.*, 1987). As stated in Chapter 1 the 126 kDa and 183 kDa replicase proteins are encoded by nucleotides 69-3419 and 69-4919 of the TMV-U1 genome, respectively (Goelet *et al.*, 1982). The latter is expressed following readthrough of an amber stop codon situated at nucleotides 3417 (Section 1.6.5). In addition to the replicase protein genes, the replication cassette also included the coding region for the putative 54 kDa protein (nucleotides 3495-4919 of the TMV-U1 genome). To date this protein expressed from subgenomic I_1-RNA (Sulzinski *et al.*, 1985) has not been detected in TMV infected tissue (Section 1.6.6). However, it has been suggested that the protein may be involved with the regulation of replication (Carr *et al.*, 1992; Ogawa *et al.*, 1992). Expression of the replicase genes by the replication cassette should have been enhanced by the 68 nucleotide TMV-U1 5' untranslated region, known as Ω (Section 1.6.3). This was known to be highly efficient 5' leader sequence (Gallie *et al.*, 1987a)

and is also necessary for viral replication (Takamatsu *et al.*, 1991).

A number of aspects relating to the design of the marker cassettes required consideration. All three marker cassettes pCS4-L1, pCSLU and pCSLLUTR should have contained an identical coat protein subgenomic promoter fused to the 5' end of the luciferase gene. Studies of BMV have provided evidence concerning the way in which subgenomic RNAs are produced (Section 1.6.6; Miller *et al.*, 1985). Analysis of the BMV coat protein subgenomic promoter has since been carried out (French and Ahlquist, 1988; Marsh *et al.*, 1988). It led to the identification of sequences, upstream from the start codon, which were thought to be functionally important. Although similar in some respects, less is known about TMV coat protein subgenomic promoters. The 98 nucleotides immediately upstream from the TMV-L coat protein start codon are known to be sufficient for subgenomic RNA production (Meshi *et al.*, 1987). However longer promoters consisting of >200 nucleotides are thought to be more efficient (Dawson *et al.*, 1989; Dawson and Lehto, 1990; Ogawa *et al.*, 1992). The presence of *cis*-acting factors within the 30 kDa protein and coat protein genes have not been associated with viral replication (Meshi *et al.*, 1987). However certain sequences may be important for regulating either the production of subgenomic RNAs or protein expression from the RNAs. In particular, it has been suggested that the 5' end of the 30 kDa protein ORF (nucleotides 4938-5263 of the TMV-L genome) may be involved with regulating the production of coat protein (Ogawa *et al.*, 1992).

The primers 5P-1 and 5P-2N were designed to amplify 224 nucleotides from the region upstream from the TMV-U1 coat protein start codon. The nucleotide immediately upstream of the start codon was replaced by two foreign nucleotides. The amplification procedure introduced three mutations at positions equivalent to nucleotides 5487, 5585 and 5705 of the TMV-U1 genome. The first two were situated >100 nucleotides upstream from the start codon. As a result, it was thought that they would have little effect on promoter function. The third was considered to be more serious, situated only 7 nucleotides upstream from the start codon it was likely to be in an important region of the subgenomic promoter. Evidence suggests that this region may be required to initiate RNA synthesis at the correct site (Section 1.6.6; Marsh *et al.*, 1988). The substitution was also within the translational leader sequence. Consequently this mutation together with the two residues introduced at the 3' end of the subgenomic promoter significantly altered the leader sequence and the context of the start codon. The subgenomic mRNA from which the coat protein is expressed has been designated LMC-RNA (Section 1.6.6). The authentic LMC-RNA leader sequence has a 5' m^7GpppG cap (Section 1.4.2) and consists of 9 residues, GUUUUAAAUAUGU. Although not common to eukaryotic mRNAs, this A/U rich region, is characteristic of

plant viral mRNAs (Goelet *et al.*, 1982). The equivalent sequence upstream from the luciferase gene start codon was GUGUUAAACCATGG. This 10 nucleotide sequence contained a lower proportion of A/U residues than the authentic TMV-U1 coat protein leader sequence. Two models have been put forward which describe the optimum start codon context for translation by eukaryotic ribosomes (Kozak, 1984; Lütcke *et al.*, 1987). The start codon context of many plant virus proteins, including the TMV-U1 coat protein, do not fit these models. However these proteins are still expressed at high levels. While the context of the luciferase gene start codon (engineered into the marker cassette) did not match the context of the TMV-U1 coat protein start codon, it did conform to the optimum models proposed by both Kozak (1984) and Lütcke *et al.* (1987). Its ability to support efficient expression of the luciferase gene remains uncertain. Lehto and Dawson (1990) attempted to improve the start context of the TMV 30 kDa protein by replacing it with sequences which conformed to the two models. No improvement was observed and in one case expression decreased. It has been suggested that expression of viral genes from subgenomic RNAs is instead enhanced by close proximity to the 3' end of the genome (French and Ahlquist, 1988; Culver *et al.*, 1993).

Sequence data shows that the coat protein terminates at nucleotide 6191 of the TMV-U1 genome leaving 204 untranslated nucleotides at the 3' end. Evidence suggesting the importance of this region in viral replication has been presented in Section 1.6.4. The region contains a distinct secondary structure which is thought to be recognised by the TMV replicase proteins (Guilley *et al.*, 1979, Reitveld *et al.*, 1984; van Belkam *et al.*, 1985). In particular a t-RNA like structure has been found aminocylated with histidine (Oberg and Philipson, 1972). Experiments conducted by Dawson *et al.* (1988) suggested that the final 175 nucleotides of the TMV-U1 genome are sufficient for viral replication. We decided to amplify the entire 3' TMV-U1 untranslated region for use in construction of the marker cassette pCSLU, together with 20 nucleotides from the 3' end of the coat protein ORF. Three alterations to the TMV-U1 sequence were introduced by the primer 3P-1. All three were within the coat protein ORF and were not considered to be important. One base substitution was introduced by the primer 3P-2, this was situated at the position equivalent to nucleotide 6837 of the TMV-U1 genome. It involved a T being substituted for A, T is the authentic base in the equivalent position of the TMV-L genome. It was thought that the substitution would be tolerated. This view was supported by Meshi *et al.* (1986), they showed that the reverse substitution introduced into a cDNA clone of TMV-L did not appear to compromise infectivity. The marker cassette pSLLUTR contained a longer region cloned from the 3' end of the TMV-U1 genome. The region included 175 nucleotides from the coat protein ORF as well as the 3' untranslated region as far

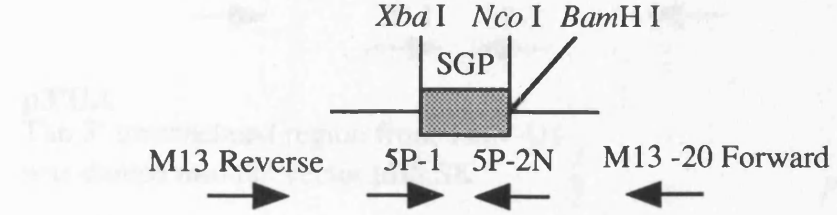
as nucleotide 6390. It was thought that the longer TMV 3' region may contain important yet undefined sequences involved with the regulation of replication or the production of subgenomic RNA. Any benefit acquired from these sequences was likely to have been masked due to deletion of the terminal 5 nucleotides from the 3' untranslated region (6391-6395). Viral replicase proteins lack proof reading mechanisms and as a result sequences must be maintained by strong selection pressures. It is unlikely that the terminal 5 nucleotides would be maintained in the TMV-U1 genome if they were not either essential for replication or had some other important function. Furthermore the secondary structure proposed by Reitveld *et al.* (1984) begins at the 3' end of the genome, with nucleotide 6391 being incorporated into the stem of a hairpin loop.

The importance of limiting non-viral nucleotides from the 3' end of transcripts has been discussed (Sections 1.4.2; 1.4.3; Boyer and Haenni, 1994). This could have be achieved by inserting a transcription termination sequence and polyadenylation signal at the 3' end of the marker cassettes. Alternatively a self cleaving ribozyme sequence could have been used. It is not known whether such sequences would have improved expression of the luciferase gene by the replication-marker construct pU-SLU. Linearisation of pCU-SLU downstream from the 3' untranslated region prior to microprojectile bombardment would have reduced the number of 3' non-viral nucleotides transcribed. However, following linearisation no improvement in luciferase activity was observed. The construct pB-35STMV was highly infectious when used to transform *N. tabacum* SR1 plants (Section 3.2.6), despite the fact that the ribozyme sequence had been removed. It was thought that likewise replication-dependent expression of the luciferase gene may have been observed if the construct from pBU-SLU was present in every cell of a plant.

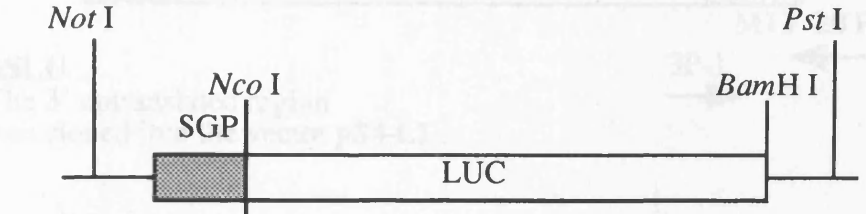
The final experiment described in Section 4.2.6.3 involved manually inoculating the transgenic line L, T and C plants with purified TMV particles. The aim was to determine whether the plants were resistant to infection (Section 1.6.6). Donson *et al.* (1993) reported that transgenic plants expressing a deleted replicase protein showed some resistance to TMV. A similar result in any of the transgenic plant lines may have suggested that the replicase proteins were not being correctly expressed. Expression of the 54 kDa protein in transgenic plants has also been associated with a resistant phenotype (Golemboski *et al.*, 1990; Carr *et al.*, 1992). Resistance to TMV in line T plants may therefore have suggested that the replication-marker construct was capable expressing the 54 kDa protein via production of subgenomic RNA. Of course there may have been another explanation behind any resistant phenotypes. The preliminary results presented in Section 4.2.6.3 are not sufficient to draw conclusions confirming

the resistant nature of the transgenic plants.

The apparent inability of the replication-marker construct pU-SLU, to express luciferase from subgenomic RNA was disappointing. Details concerning design and construction of the replicase-marker construct, pU-S4-L1 and replication-marker constructs, pU-SLU and pU-SLLUTR, have been discussed. As a result a number of features which may had led to compromised activity have been identified. Without further analysis of the transgenic plants, definite conclusions regarding the significance of these features cannot be made. Having carefully considered the points raised in this discussion it was decided to generate two new and hopefully improved replication-marker constructs. The design and transient analysis of these constructs has been described in Chapter 5.



pSGP4
The TMV-U1 coat protein subgenomic promoter
was cloned into the vector pBS SK-



a) GTGTTAACC ATG
b) GTTTTAAAT

SGP-LUC fusion

pS4-L1
The luciferase gene
was cloned into the vector pSGP4

Figure 4.1 Diagrams of pSGP4 and the Marker Cassette pS4-L1.
The restriction enzyme sites and primer binding sites used during construction have been indicated. The nine nucleotides upstream of the ATG luciferase start codon have also been indicated (a), together with the authentic TMV-U1 coat protein untranslated leader sequence (b).

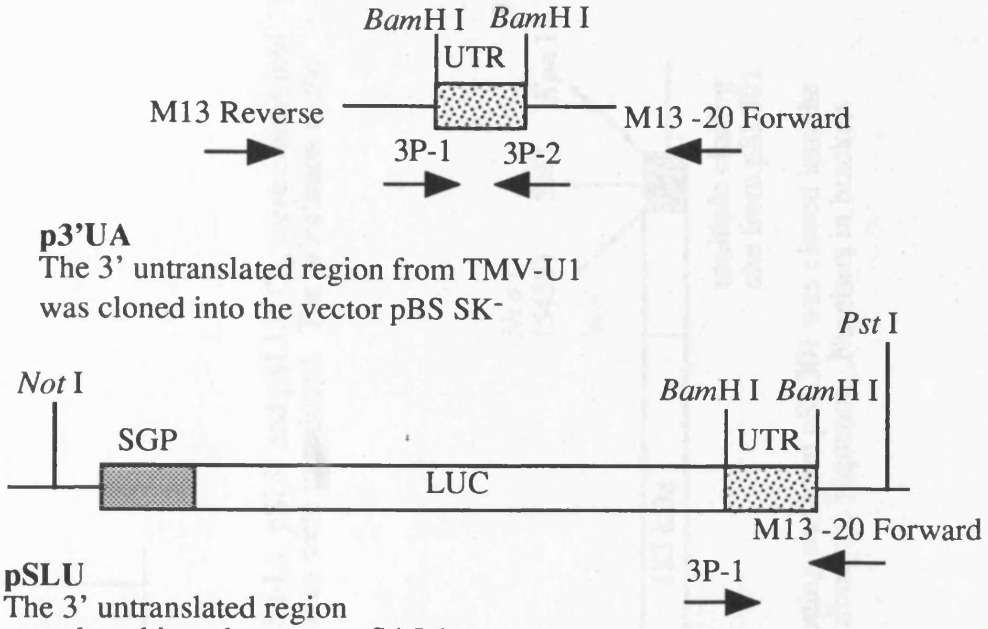


Figure 4.2 Diagrams of p3'UA and the Marker Cassette pSLU.
PCR amplification using primers 3P-1 and M13 -20 Forward allowed the TMV-U1 3' untranslated region to be orientated within pSLU.

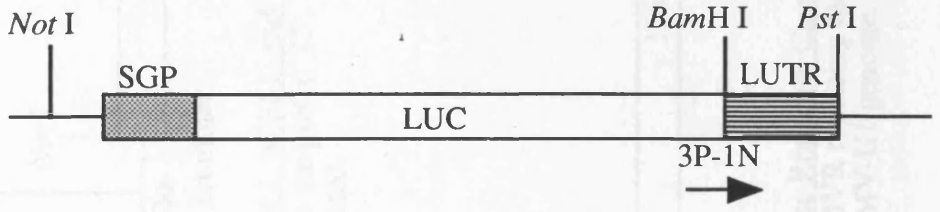


Figure 4.3 Diagram of the Marker Cassette pSLLUTR.

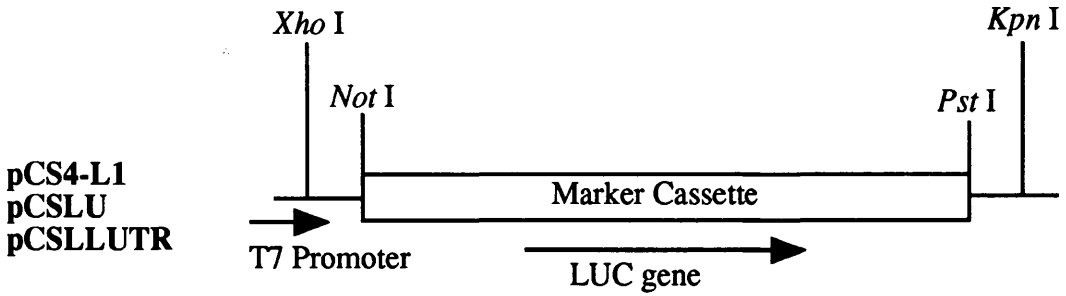


Figure 4.4 Diagram of pCS4-L1, pCSLU and pCSLLUTR. The marker cassettes, pS4-L1, pSLU and pSLLUTR, were cloned into the vector pcDNA II and given the prefix "C". As a result useful restriction enzyme sites were introduced. The orientation of the luciferase gene has been indicated.

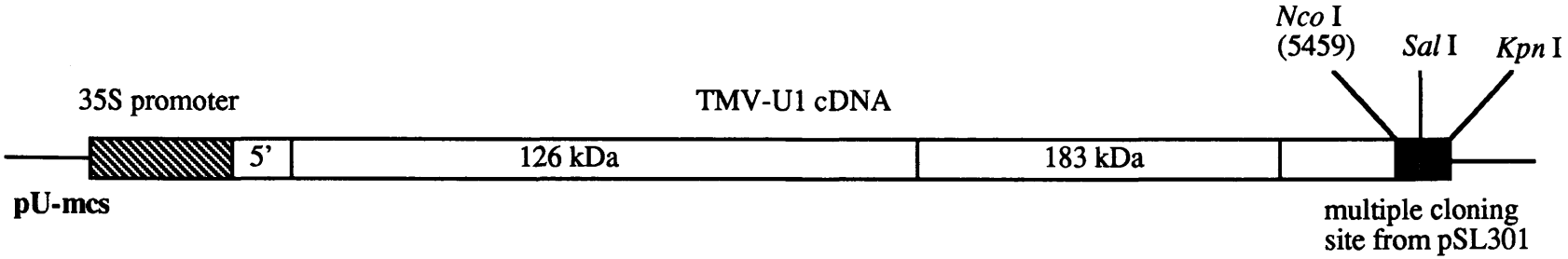


Figure 4.5 Diagram of the Replicase Cassette pU-mcs. Part of the multiple cloning site from pSL301 was cloned into the infectious construct p35STMVR replacing the 3' end of the TMV genome and the ribozyme sequence. Numbers in brackets refer to nucleotides of the TMV-U1 genome.

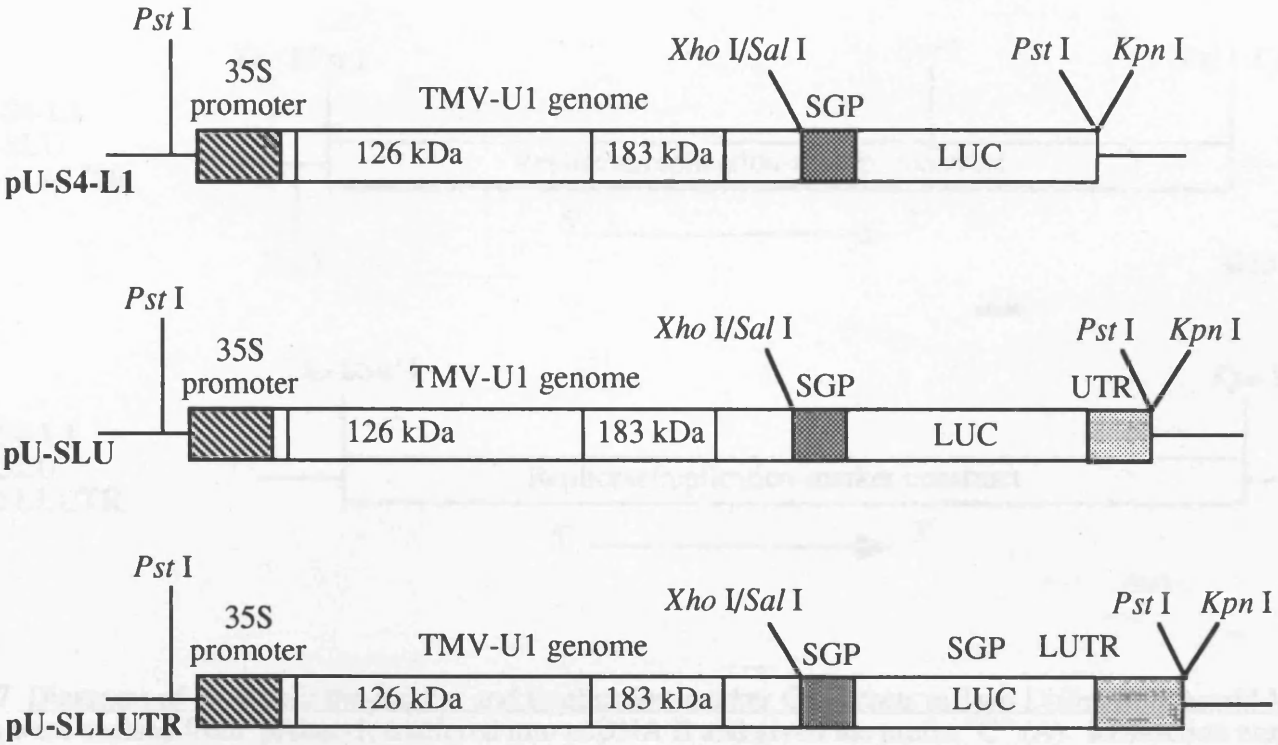


Figure 4.6 Diagrams of the Replicase-marker Construct pU-S4-L and Replication-marker Constructs pU-SLU and pU-SLLUTR. The marker cassettes were independently joined to the replicase cassette pU-mcs.

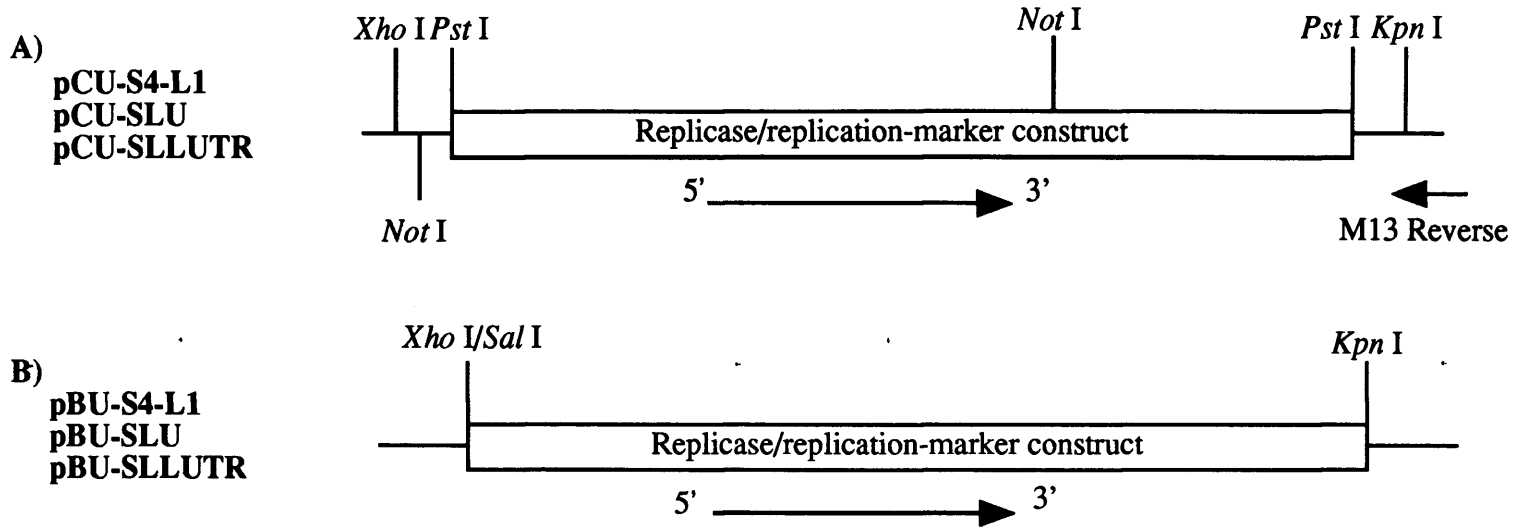
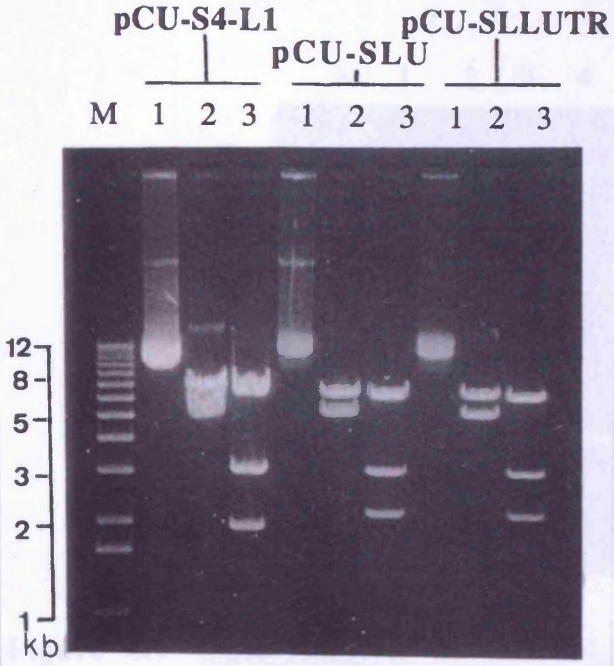


Figure 4.7 Diagrams of the Replicase-marker and Replication-marker Constructs in Two Different Plasmid Vectors. The constructs were excised from pAlter-1, tranfered into pcDNA II and given the prefix "C" (A). Restriction enzymes sites used for diagnosis as well as cloning have been indicated (see Figure 4.8). The constructs were transferred to the binary vector pBin 19 and given the prefix "B" (B). The arrows indicate the orientation of the TMV-U1 genome and luciferase gene within the vectors.

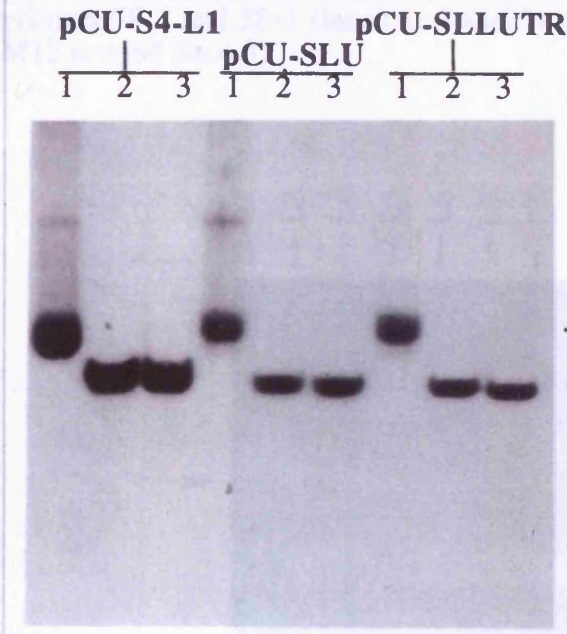
A)



B)

Lane	Enzyme	Expected fragment size
2	*Not* I	6.2 kb[c], 4.8-5.2 kb[d]
3	*Not* I, *Pst* I	6.2 kb[c], 2.9 kb, 1.9-2.3 kb[d]

C)



D)

Figure 4.8 Restriction Enzyme Digests of pCU-S4-L1, pCU-SLU and pCU-SLLUTR. DNA fragments were visualised on an agarose gel (A). Molecular weight markers (M), undigested DNA (lanes 1). Table showing restriction enzymes and expected fragment sizes (B), exact fragment sizes were dependent upon the presence and size of the TMV 3' region. Southern blot of (A) probed with an ~700 bp *Sst* II, *Bam*H I fragment isolated from pC-35STMVR (C), in (B) [c] indicates fragments to which the probe hybridised. Southern blot of (A) probed with an ~750 bp *Eco*R I, *Eco*R V luciferase gene fragment isolated from pRTS2-LUC (D), in (B) [d] indicates fragments to which the probe hybridised. The blots were exposed to X-ray film for 4 hours.

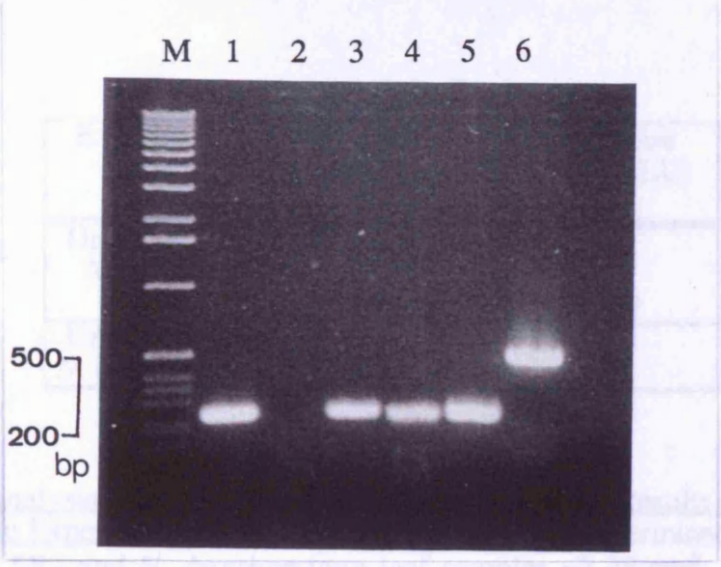


Figure 4.9 PCR Analysis of pCU-S4-L1, pCU-SLU and pCU-SLLUTR. Molecular weight markers (M). PCR amplification from the replicase-marker construct pCU-S4-L1 (lanes 1 and 2); replication-marker constructs pCU-SLU (lanes 3 and 4) replication-marker construct pCU-SLLUTR (lanes 5 and 6). DNA amplified using the primers 5P-1 and 5P-1 (lanes 1, 3 and 5); 3P-1 and 3P-2 (lanes 2 and 4), 3P-1N and M13 reverse (lane 6).

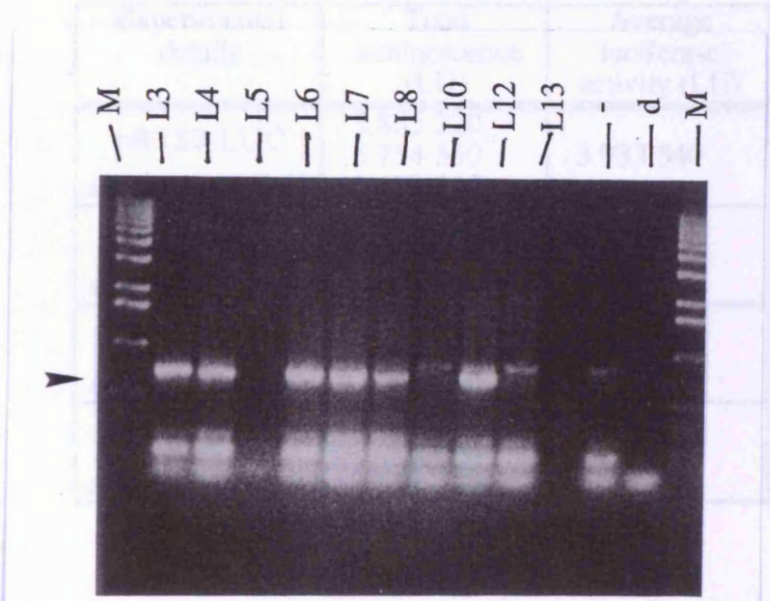


Figure 4.10 PCR Analysis of *N. tabacum* SR1 Line L Plants. The primers 5'REP and 3'REP were used to amplify a 735 bp fragment from line L plants transformed with the replicase-marker construct form pBU-S4-L1 Molecular weight markers (M). PCR using genomic DNA prepared from an untransformed *N. tabacum* SR1 plant (-); sample DNA replaced by dH_2O (d). PCR amplified fragments are indicated by the arrow.

Experimental details	Total luminescence (LU)	Luciferase activity (LU)
Unbombarded *N. tabacum* SR1	6330	430
	5760	1160
Unbombarded *N. benthamiana*	2820	0

Table 4.1 Analysis of the Replication-marker Constructs: Results of Microprojectile Bombardment Experiment 4.1. Luciferase activities were determined for unbombarded *N. tabacum* SR1 and *N. benthamiana* leaf samples ~2.25 cm^2. The sample were detached from plants immediately prior to performing the assays.

Experimental details	Total luminescence (LU)	Average luciferase activity (LU)
pRTS2-LUC assayed at 1 dpb.	3 885 220 5 754 350 2 171 850	3 933 540
pCU-S4-L1 assayed at 1 dpb.	3 480 5 740 7 720	0
pCU-SLU assayed at 1 dpb.	6 250 6 330 6 780	2 053
pCU-SLLUTR assayed at 1 dpb.	3 220 4 040 4 320	0

Table 4.2 Analysis of the Replication-marker Constructs: Results of Microprojectile Bombardment Experiment 4.2. *N. tabacum* SR1 leaf samples ~2.25 cm^2 were assayed at 1 day post bombardment (dpb) with either pCU-S4-L1, pCU-SLU or pCU-SLLUTR.

Experimental details	Total luminescence (LU)	Average luminescence	Average GUS activity (FU min^{-1})
pCU-S4-L1 assayed at 3 dpb.	2 260 2 250 3 350 2 800 2 280 2 560	2 583	53.73
pCU-SLU assayed at 3 dpb.	4 020 2 130 2 490 2 920 3 390 2 580	2 922	12.26
pCU-SLLUTR assayed at 3 dpb.	2 750 2 810 2 770 3 070 2 360 2 620	2 730	42.21

Table 4.3 Analysis of the Replication-marker Constructs: Results of Microprojectile Bombardment Experiment 4.3. *N. tabacum* SR1 leaf samples ~2.25 cm^2 were assayed at 3 days post bombardment with either pCU-S4-L1, pCU-SLU or pCU-SLLUTR. The construct pRTL2-GUS was included as an internal control to determine the success of bombardments.

Experimental details	Total luminescence (LU)	Average luciferase activity (LU)	Average GUS Activity (FU min^{-1})	Relative luciferase activity
pCU-S4-L1 *Xho* I digested, assayed at 3 dpb.	1 670 1 540 1 560 1 610 1 770 2 260	0	0.91	0
pCU-SLU *Xho* I digested, assayed at 3 dpb.	2 490 2 480 2 240 2 900 5 810 2 900	970	0.85	1 141
pCU-SLLUTR *Xho* I digested, assayed at 3 dpb.	2 390 3 290 2 820 2 120 2 680 2 350	375	0.89	421

Table 4.4 Analysis of the Replication-marker Constructs: Results of Microprojectile Bombardment Experiment 4.4. *N. tabacum* SR1 leaf samples ~2.25 cm^2 were assayed at 3 days post bombardment with either pCU-S4-L1, pCU-SLU or pCU-SLLUTR. The constructs were digested using *Xho* I prior to bombardment. This linearised the plasmids downstream from the luciferase gene and TMV 3' untranslated region. The construct pRTL2-GUS was included as an internal control to determine the success of bombardments.

Experimental details	Total luminescence (LU)	Average luciferase activity (LU)	Average GUS activity (FU min^{-1})	Relative luciferase activity
pCU-S4-L1 *Xho* I digested, assayed at 1 dpb.	4 450 9 870	0	59.70	0
pCU-SLU *Xho* I digested, assayed at 1 dpb.	9 770 8 090	0	71.10	0
pCU-SLLUTR *Xho* I digested, assayed at 1 dpb.	7 600 5 100	0	60.20	0

Table 4.5 Analysis of the Replication-marker Constructs: Results of Microprojectile Bombardment Experiment 4.5. *N. tabacum* SR1 leaf samples ~2.25 cm^2 were assayed at 1 day post bombardment with either pCU-S4-L1, pCU-SLU or pCU-SLLUTR. The constructs were digested using *Xho* I prior to bombardment. This linearised the plasmids downstream from the luciferase gene and TMV 3' untranslated region. The construct pRTL2-GUS was included as an internal control to determine the success of bombardments.

Experimental details	Total luminescence (LU)	Average luciferase activity (LU)	Average GUS activity (FU min^{-1})	Relative luciferase activity
pCU-S4-L1 *Xho* I digested, assayed at 3 dpb.	7 620 7 280 8 550 13 390	3 210	16.77	191.4
pCU-SLU *Xho* I digested, assayed at 3 dpb.	9 190 7 560 10 610 13 610	3 443	39.60	86.9
pCU-SLLUTR *Xho* I digested, assayed at 3 dpb.	8 840 10 280 10 200 6 250	2 893	27.50	105.2

Table 4.6 Analysis of the Replication-marker Constructs: Results of Microprojectile Bombardment Experiment 4.6. *N. tabacum* SR1 leaf samples ~2.25 cm^2 were assayed at 3 days post bombardment with either pCU-S4-L1, pCU-SLU or pCU-SLLUTR. The constructs were digested using *Xho* I prior to bombardment. This linearised the plasmids downstream from the luciferase gene and TMV 3' untranslated region. The construct pRTL2-GUS was included as an internal control to determine the success of bombardments.

Experimental details	Total luminescence (LU)	Average luciferase activity (LU)	Average GUS activity (FU min^{-1})	Relative luciferase activity
pCU-S4-L1	4 100			
Xho I digested,	5 580	0	52.68	0
assayed at 6 dpb.	4 530			
pCU-SLU	5 450			
Xho I digested,	4 650	0	57.12	0
assayed at 6 dpb.	4 580			
pCU-SLLUTR	4 110			
Xho I digested,	5 360	0	38.43	0
assayed at 6 dpb.	4 700			

Table 4.7 Analysis of the Replication-marker Constructs: Results of Microprojectile Bombardment Experiment 7. *N. tabacum* SR1 leaf samples ~2.25 cm^2 were assayed at 6 days post bombardment with either pCU-S4-L1, pCU-SLU or pCU-SLLUTR. The constructs were digested using *Xho* I prior to bombardment. This linearised the plasmids downstream from the luciferase gene and TMV 3' untranslated region. The construct pRTL2-GUS was included as an internal control to determine the success of bombardments.

Experimental details	Total luminescence (LU)	Average luciferase activity (LU)	Average GUS activity (FU min^{-1})	Relative luciferase activity
pCU-S4-L1 *Pst* I	5 520			
digested and		0	89.05	0
p35STMVR,	3 560			
assayed at 2 dpb.	4 380			
pCU-SLU *Pst* I	4 050			
digested and		0	49.85	0
p35STMVR	4 840			
assayed at 2 dpb.	5 580			
pCU-SLLUTR	5 880			
Pst I digested		260	96.15	2.70
and p35STMVR,	5 000			
assayed at 2 dpb.	6 100			

Table 4.8 Analysis of the Replication-marker Constructs: Results of Microprojectile Bombardment Experiment 4.8. *N. tabacum* SR1 leaf samples ~2.25 cm^2 were assayed at 2 days post bombardment with the infectious construct p35STMVR and either pCU-S4-L1, pCU-SLU or pCU-SLLUTR. The constructs were digested using *Pst* I prior to bombardment. This resulted in the constructs being excised from the pcDNA II vector. The construct pRTL2-GUS was included as an internal control to determine the success of bombardments.

Experimental details	Total luminescence (LU)	Average luciferase activity (LU)
pCU-S4-L1 *Pst* I digested and p35STMVR, assayed at 4 dpb.	3 670 4 360 3 460	0
pCU-SLU *Pst* I digested and p35STMVR assayed at 4 dpb.	3 760 3 880 4 720	0
pCU-SLLUTR *Pst* I digested and p35STMVR, assayed at 4 dpb.	3 880 2 830 4 450	0

Table 4.9 Analysis of the Replication-marker Constructs: Results of Microprojectile Bombardment Experiment 9. *N. tabacum* SR1 leaf samples ~2.25 cm^2 were assayed at 4 days post bombardment with the infectious construct p35STMVR and either pCU-S4-L1, pCU-SLU or pCU-SLLUTR. The constructs were digested using *Pst* I prior to bombardment. This resulted in the constructs being excised from the pcDNA II vector.

Experimental details	Total luminescence (LU)	Average luciferase activity (LU)
pCU-S4-L1 *Pst* I digested and p35STMVR, assayed at 4 dpb.	5 860 5 900 2 880	280
pCU-SLU *Pst* I digested and p35STMVR assayed at 4 dpb.	3 850 7 770 5 070	0
pCU-SLLUTR *Pst* I digested and p35STMVR, assayed at 4 dpb.	4 910 5 050 4 390	0

Table 4.10 Analysis of the Replication-marker Constructs: Results of Microprojectile Bombardment Experiment 4.10. *N. tabacum* SR1 leaf samples ~2.25 cm^2 were assayed at 4 days post bombardment with the infectious construct p35STMVR and either pCU-S4-L1, pCU-SLU or pCU-SLLUTR. The constructs were digested using *Pst* I prior to bombardment. This resulted in the constructs being excised from the pcDNA II vector.

Experimental details	Total luminescence (LU)	Average luciferase activity (LU)
pCU-S4-L1 *Pst* I digested and p35STMVR, assayed at 4 dpb.	2 010 1 590 4 540	1 046
pCU-SLU *Pst* I digested and p35STMVR assayed at 4 dpb.	1 420 1 960 2 670	217
pCU-SLLUTR *Pst* I digested and p35STMVR, assayed at 4 dpb.	1 560 2 020 2 120	0

Table 4.11 Analysis of the Replication-marker Constructs: Results of Microprojectile Bombardment Experiment 4.11. *N. benthamiana* leaf samples ~2.25 cm^2 were assayed at 4 days post bombardment with the infectious construct p35STMVR and either pCU-S4-L1, pCU-SLU or pCU-SLLUTR. The constructs were digested using *Pst* I prior to bombardment. This resulted in the constructs being excised from the pcDNA II vector.

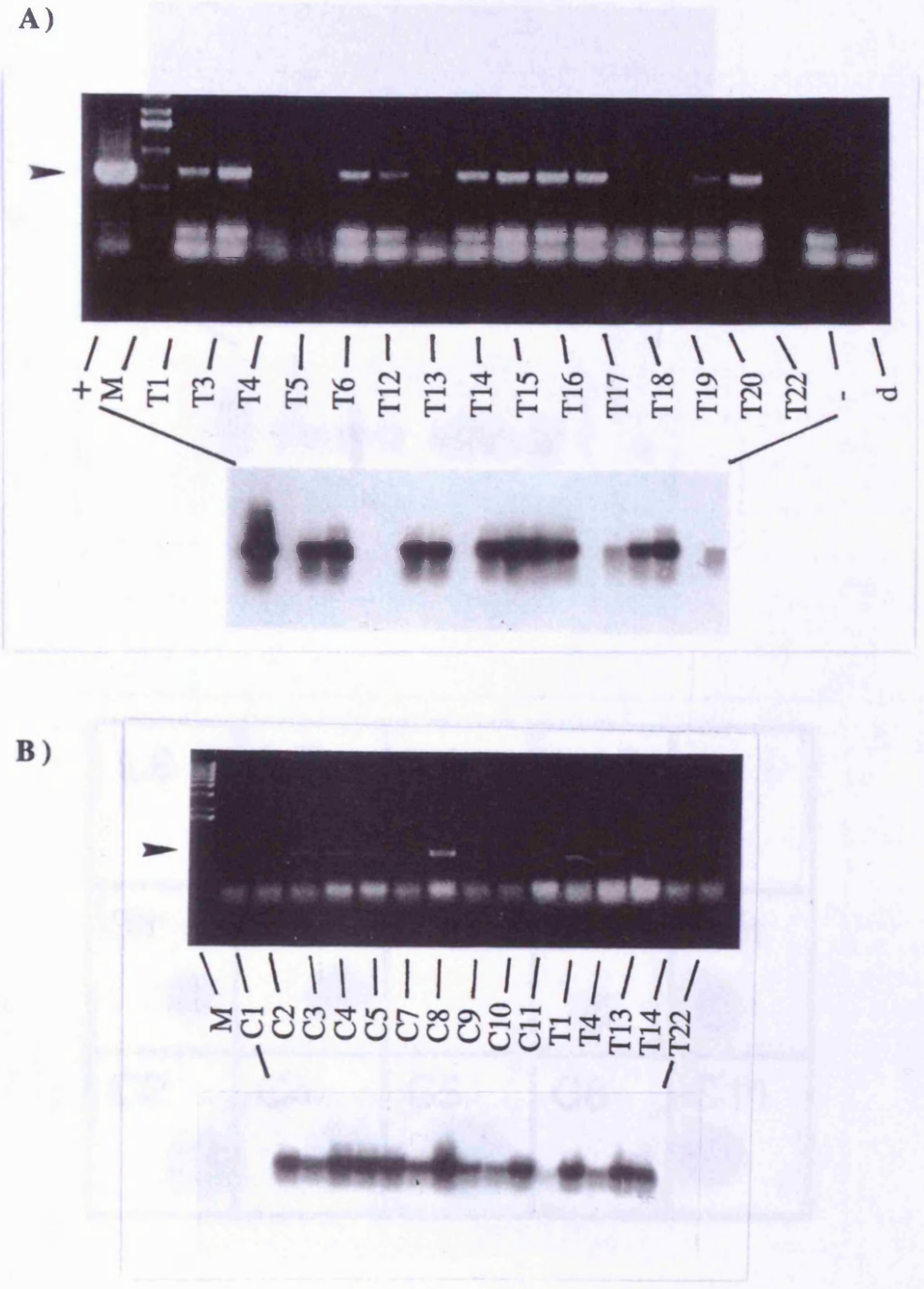


Figure 4.11 PCR Analysis of *N. tabacum* SR1 Line T and Line C Plants. The primers 5'REP and 3'REP were used to amplify a 735 bp fragment from line T plants transformed with the replication-marker construct from pBU-SLU; line C plants transformed with the replication-marker construct from pBU-SLLUTR. Molecular weight markers (M). PCR using genomic DNA prepared from an untransformed *N. tabacum* SR1 plant (-); sample DNA replaced by dH_2O (d). PCR amplified fragments are indicated by the arrow. Agarose gels were Southern blotted and probed with an ~700 bp *Sst* II, *Bam*H I fragment isolated from pC-35STMVR. Blots were exposed to X-ray film for 4 hours.

Figure 4.11 C)

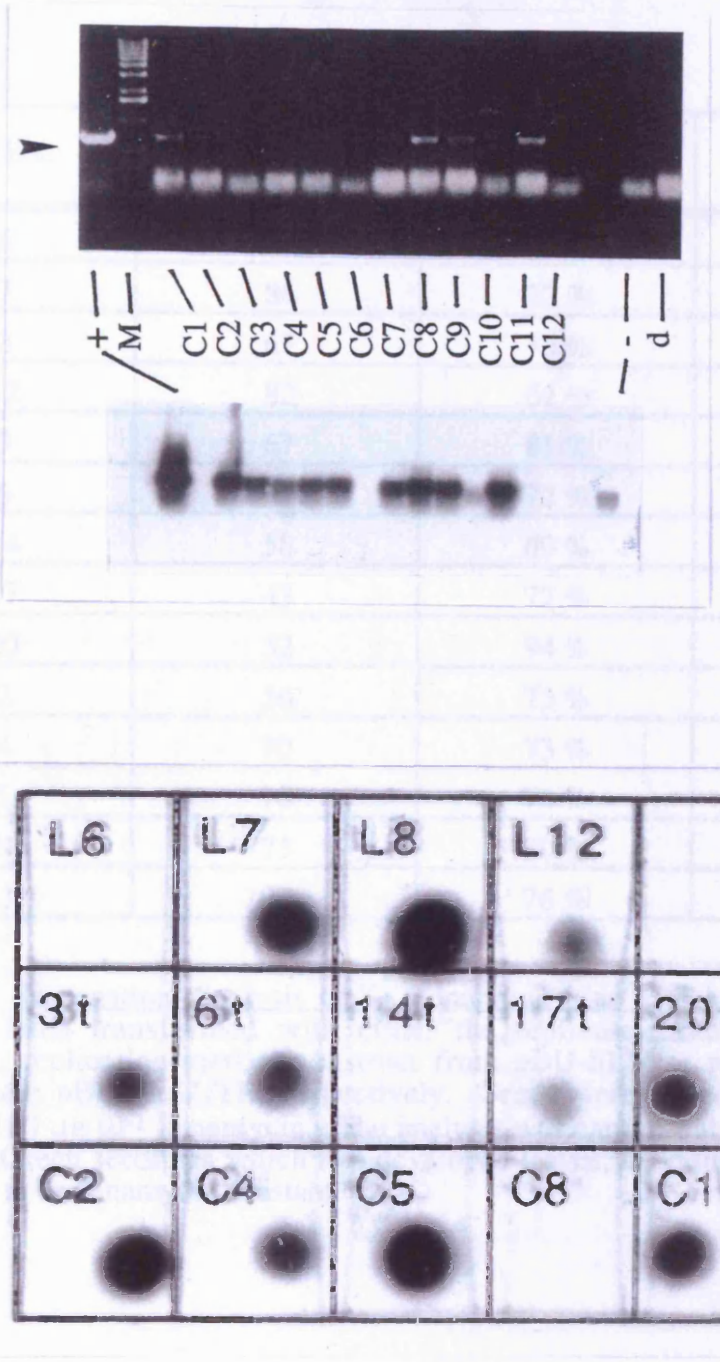


Figure 4.12 Dot Blot to Detect TMV RNA in TMV Inoculated Line L. T. and C Plants. T_1 generation plants were inoculated with ~5 µg of purifies TMV particles. Extracts were prepared from uninoculated leaves at 7 days post inoculation. The Blot was probed with an ~3.6 kb *Hind* III fragment isolated from pC-35STMVR and exposed to X-ray film for 24 hours.

Plant line	Number of seeds to germinate	Percentage resistant to kanamycin	Percentage susceptible to kanamycin
L6	66	94 %	6 %
L7	86	72 %	28 %
L8	67	75 %	25 %
L12	82	62 %	38 %
T3	67	81 %	19 %
T6	69	72 %	28 %
T14	58	69 %	31 %
T17	43	77 %	23 %
T20	52	94 %	6%
C2	56	75 %	25 %
C4	70	73 %	27 %
C5	70	74 %	26 %
C8	75	80 %	20 %
C11	107	76 %	24 %

Table 4.12 Segregation Analysis of T_1 Generation Line L, T and C Plants. The plants had been transformed with either the replicase-maker construct from pBU-S4-L1, replication-marker construct from pBU-SLU or replication-marker construct from pBU-SLLUTR, respectively. Seeds were plated on MSO media containing 100 $\mu g\ \mu l^{-1}$ kanamycin. The analysis was carried out on four week old seedlings. Green seedlings which had developed leaves, in addition to cotyledons, were judged to be kanamycin resistant.

Plant Line	Luciferase activity (LU mg protein^{-1})	Average Luciferase activity (LU mg protein^{-1})
L6	14 144 0	7 072
L7	18 223 8 491	13 357
L8	8 377 1 364	4 870
L12	4 291 5 939	5 115
T3	10 907 8 374	9 640
T6	30 073 13 651	21 862
T14	16 542 2 783	9 662
T17	30 016 13 036	21 526
T20	28 426 32 014	30 220
C2	40 018 37 139	38 578
C4	390 3 088	1 739
C5	15 256 11 581	13 418
C8	14 783 9 696	12 239
C11	9 833 5 531	7 682

Table 4.13 Luciferase Expression by T_1 Generation "A" Plants. The line L, T and C plants should have been expressing the replicase-maker construct from pBU-S4-L1, replication-marker construct from pBU-SLU or replication-marker construct from pBU-SLLUTR, respectively. Two samples ~2.25 cm^2 were removed from the plants. Each was assayed to determine its luciferase activity and protein concentration.

Plant Line	Luciferase activity (LU mg protein^{-1})	Average Luciferase activity (LU mg protein^{-1})
L6	5 641 720	3 180
L7	38 571 46 421	42 496
L8	4 501 2 606	3 553
L12	59 679 12 134	35 906
T3	6 179 13 247	9 713
T6	28 725 10 500	19 612
T14	19 978 5 886	12 932
T17	18 484 5 376	11 930
T20	25 681 3 206	14 443
C2	30 628 14 932	22 780
C4	15 402 7 002	11 202
C5	30 523 7 155	18 839
C8	6 614 6 016	6 315
C11	89 979 16 069	53 024

Table 4.14 Luciferase Expression by T_1 Generation "B" Plants. The line L, T and C plants should have been expressing the replicase-maker construct from pBU-S4-L1, replication-marker construct from pBU-SLU or replication-marker construct from pBU-SLLUTR, respectively. Two samples ~2.25 cm^2 were removed from the plants. Each was assayed to determine its luciferase activity and protein concentration.

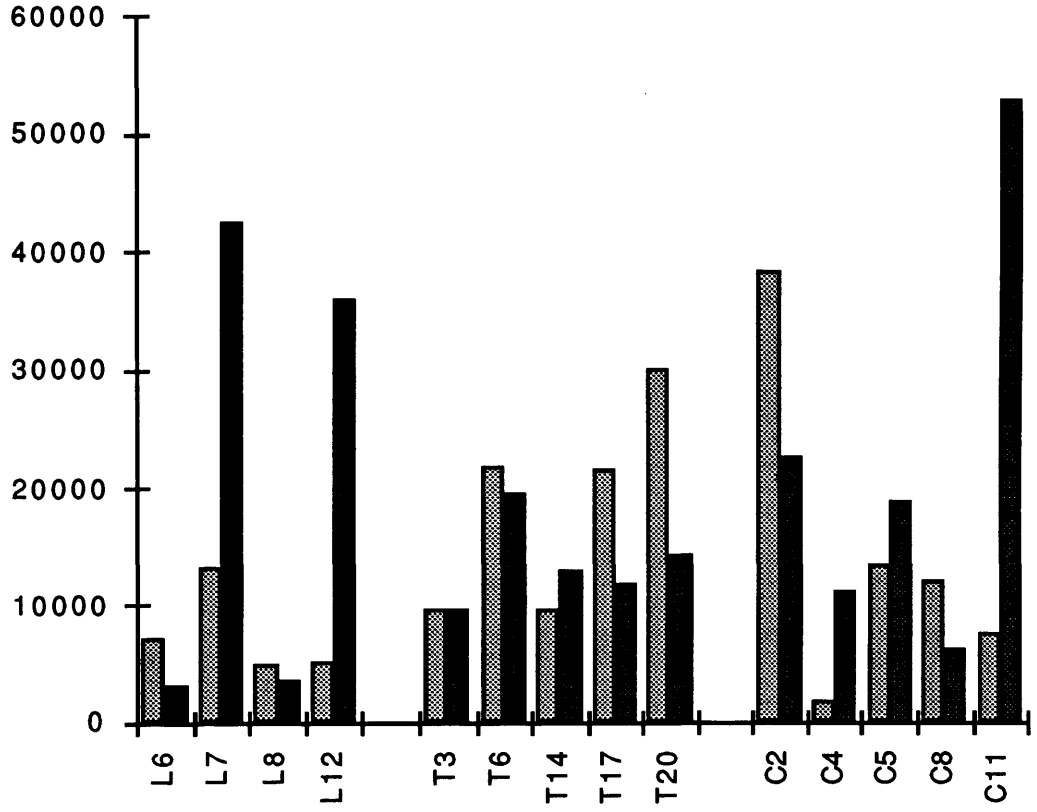


Figure 4.13 Bar Chart Comparing Data Presented in Tables 4.13 and 4.14. Average luciferase activities were calculated following the analysis of two samples ~2.25 cm^2 from T_1 generation line L, T, and C plants. "A" plants (light shade on left); "B" plants (dark shade on right). Y axis equals LU mg $protein^{-1}$.

CHAPTER 5

THE GENERATION OF TWO NEW REPLICATION-MARKER CONSTRUCTS

5.1 INTRODUCTION

The main aim of this project was to develop a TMV-based expression vector which would allow replication-dependent expression of a marker gene (Sections 1.10; 4.1). This was to be achieved by placing the luciferase gene downstream of the TMV-U1 replicase genes and under control of the coat protein subgenomic promoter. The design and analysis of two replication-marker constructs has been described in Chapter 4. Microprojectile bombardment experiments failed to detect luciferase activities significantly higher than background luminescences in samples bombarded with the replication-marker constructs. The luciferase activities detected in plants which had been transformed with the replication-marker constructs were slightly higher than background luminescence. However, similar activities were also detected in plants transformed with a control replicase-marker construct. RNA transcribed by this construct was designed to be incapable of replication. The design of the constructs has been critically analysed in Section 4.3, the result of this analysis have been summarised below.

i) The replication cassette consisted of the 35S promoter fused to the 5' region of the TMV-U1 genome, up to nucleotide 5459. It was obtained from the highly infectious construct p35STMVR (see Section 3.2). Mutations within the replication cassette can not be ruled out. However, there is no evidence to suggest that viable replicase proteins were not expressed. The results of *in vitro* luciferase gene analysis suggested that this gene sequence was also viable.

ii) It has not been confirmed whether *cis*-acting factors exist within the 30 kDa protein and coat protein ORFs. Such factors may be associated with the regulation of protein expression from sub-genomic mRNAs. It has been suggested that the 5' region of the 30 kDa ORF may be involved regulating the production of coat protein (Ogawa *et al.*, 1992).

iii) The presence of errors in the coat protein subgenomic promoter, particularly at the 3' end, may have altered interactions between the promoter and the TMV replicase proteins. In addition, alterations to the authentic LMC-RNA untranslated leader sequence, together with the context of the ATG start codon, may have altered the efficiency of translation.

iv) There was no obvious evidence suggesting that the 3' untranslated region of pU-SLU was non-functional. A deletion at the 3' end of this region in the second

construct, pSLLUTR, suggested that RNA transcribed by the construct would have been incapable of replication (Section 1.6.4).

v) The absence of a sequence which could limit the number of non-viral nucleotides at the 3' end of transcripts was likely to have reduced the efficiency of replication (Section 1.4.2).

It was decided to build two new replication-marker constructs with the TMV-U1 coat protein gene replaced by the luciferase gene. In order to reduce the risk of errors, it was decided to keep PCR amplification to a minimum. The first construct, pTMVLUR was designed to be incapable of cell-to-cell movement. It was intended to accomplish this by introducing a frame shift mutation at the *Nco* I site, nucleotide 5459 of the TMV-U1 genome. The remaining 30 kDa protein ORF was to remain intact. It was essential that the region upstream from the coat protein ORF was recognised by the TMV replication complex (Section 1.6.6) and that the LMC-RNA 5' untranslated leader sequence interacted with host ribosomes. It was decided that, preserving the authentic LMC-RNA subgenomic promoter and leader sequence may be more important than preserving the exact context of the start codon. A PCR primer was designed to maintain this sequence and introduce a downstream *Nco I* site, to allow introduction of the luciferase gene. As a result, three nucleotides were added between the LMC-RNA leader sequence and the start codon of the luciferase gene. The authentic LMC-RNA subgenomic promoter and leader sequence was also maintained in the second construct, pTMVB-LUR. In addition, the 30 kDa cell-to-cell movement protein was left intact. The luciferase gene was inserted downstream from the coat protein ATG start codon. The two active start codons were separated by 81 nucleotides, 42 were derived from the 5' end of the coat protein gene. The remaining 39 included two inframe UAA stop codons. Both constructs contained the authentic TMV-U1 3' untranslated region and downstream ribozyme sequence, from p35STMVR (Section 3.2.1). As a result transcripts should have been cleaved leaving only four non-viral 3' nucleotides.

5.2 RESULTS

5.2.1 The Construction of pTMVLUR

5.2.1.1 Subcloning Sections of pC-35STMVR

The infectious construct pC-35STMVR has been discussed in Chapter 3. It consisted of a full length cDNA clone of TMV-U1 which was transcribed *in planta* by the 35S promoter (Figure 3.1). In order to build pTMVLUR it was necessary to

sub-clone sections of the pC-35STMVR into separate plasmid vectors. This resulted in either the removal or addition of certain restriction enzyme sites. Using *Nco* I and *Pst* I the 3' terminal 937 nucleotides of the TMV-U1 genome, together with the downstream ribozyme sequence, were excised from pC-35STMVR. Following purification they were ligated into *Nco* I, *Pst* I digested pSL301, the resulting construct was designated pSL3' (Figure 5.1). The 35S promoter fused to the first 3337 nucleotides of the TMV-U1 genome was cloned into a plasmid vector, similar to pBS SK$^-$, designated pBSXSII. In order to generate pBSXSII, pBS SK$^-$ was digested using *Sst* II. The cohesive 3' overhangs were then blunted according to the method described in Section 2.5.8 and a ligation reaction was set up to rejoin the new plasmid. Restriction enzyme digests were used to confirm that the *Sst* II site was no longer functional while the neighbouring *Not* I site remained unaltered. The construct pC-35STMVR was digested using *Not* I and *Bam*H I. The DNA fragment containing the 35S promoter and 5' end of the TMV genome was purified and ligated into similarly digested pBSXSII. The resulting construct was designated p5'TMV (Figure 5.1).

5.2.1.2 Preparation and Subcloning of pCU-XM

Removal of the *Nco* I site, situated at nucleotide 5459 of the TMV-U1 genome, had two advantages. Firstly, it allowed the luciferase gene to be linked to the coat protein subgenomic promoter using *Nco* I. Secondly, a mutation within 30 kDa protein ORF removed the cell-to-cell movement function of this protein (Section 1.7.2). The construct pC-35STMVR was digested using *Nco* I, the protocol described in Section 2.5.7 was then used to fill in the cohesive 5' overhangs. Following this a ligation reaction was set up in order to rejoin the plasmid which was designated pCU-XM (Figure 5.2). The construct was sequenced using the primer 5NG (described in Section 5.2.1.4). The result revealed that an 85 nucleotide deletion had occurred removing nucleotides 5375-5459 of the TMV-U1 genome. The origin of this deletion, which ended with the first nucleotide of the *Nco* I site (5459), remains unclear. pCU-XM was digested using *Sst* II and *Pst* I. A DNA fragment containing the 3' end of the TMV genome (including the deletion and mutated *Nco* I site) followed by the downstream ribozyme sequence was purified. The fragment was ligated into *Sst* II, *Pst* I digested pBS SK$^-$, the resulting construct was designated pSIIP3' (Figure 5.2).

5.2.1.3 The 3' Untranslated Region and Ribozyme Sequence

The TMV 3' untranslated region and downstream ribozyme sequence was cloned downstream of the luciferase gene in pS4-L1 (Section 4.2.2.1). In order to accomplish this the construct pSL3' was digested using *Bst*Y I and *Pst* I and the

~350 bp fragment was purified. This contained 273 nucleotides from the 3' end of the TMV-U1 genome together with the ribozyme sequence. The fragment was ligated into *Bam*H I , *Pst* I digested pS4-L1. It should be noted that *Bst*Y I and *Bam*H I are compatible but cannot be redigested. The correct identity of the resulting construct was confirmed by sequencing using the M13 -20 Forward primer. The construct was designated pNSLUR (Figure 5.3).

5.2.1.4 The Coat Protein Subgenomic Promoter

As explained in Section 5.1, it was decided that the authentic A/U rich LMC-RNA translation leader sequence should be maintained within the coat protein subgenomic promoter. It was also decided that, in order to reduce the risk of incorporating errors PCR amplification should be kept to a minimum. The primer 5NG, 5' GCCATGGTATTTAAAACGAATCCGATTCGGCG 3', was designed. It was complementary to nucleotides 5688-5712 of the TMV-U1 genome and included a *Nco* I site at its 5' end. The M13 Reverse primer was used in conjunction with 5NG in order amplify an ~300 bp fragment from pSL3' (Section 5.2.1.1). It included the 253 nucleotides upstream from the TMV-U1 coat protein gene. The PCR product was cloned directly into the PCR vector pGEM T, resulting in the construct pGSGP (Figure 5.4). The subgenomic promoter region was then excised from pGSGP using flanking *Nco* I sites and religated into *Nco* I digested, dephosphorylated pNSLUR. This resulted in the new coat protein subgenomic promoter being inserted upstream of the luciferase gene and downstream from the old subgenomic promoter (described in Section 4.2.2.1). PCR analysis, using the M13 Reverse and 5NG primers, was used in order to select a product containing the new subgenomic promoter in the correct orientation. A suitable construct was selected and designated pGSLUR (Figure 5.4).

5.2.1.5 Completing the Construct

The construct pGSLUR was digested using *Cla* I. An ~2 kb fragment, containing 49 nucleotides from the new coat protein subgenomic promoter and ~1.4 kb from the luciferase gene, was purified. The fragment was ligated into *Cla* I digested, dephosphorylated pSIIP3'. Restriction enzyme digests and PCR analysis, using the primers 5P-1 and 5NG, was used to select the correctly orientated product designated pT3'1/2LUC (Figure 5.5). pT3'1/2LUC was sequenced using the primer 5P-1 (Section 4.2.2.1). The result showed that all 49 nucleotides cloned as a result of PCR amplification were correct. The subgenomic promoter-luciferase gene fusion contained the authentic coat protein gene leader sequence. However, the primer 5NG introduced 3 nucleotides (ACC) between the subgenomic promoter sequence and the

luciferase gene start codon. The entire luciferase gene sequence (~1.7 kb), together with the TMV 3' untranslated region and downstream ribozyme sequence, was isolated from pGSLUR using *Nco* I and *Xho* I. The fragment was ligated into similarly digested pT3'1/2LUC, resulting in the construct pT3'LUR (see Figure 5.5). The final cloning step involved excising the new marker cassette from pT3'LUR using *Sst II* and *Xho I*. Following purification it was ligated into similarly digested p5'TMV. The completed new replication-marker construct was designated pTMVLUR (Figure 5.6).

5.2.2 The Construction of pTMVB-LUR

In order to construct pTMVB-LUR two as yet undescribed constructs were required. pSYN-LUC (Turner *et al.*, 1997) was a 35S-luciferase construct in the plasmid vector pBS SK$^-$. Its was used to provide a *Bst*X I restriction enzyme site upstream from the luciferase gene. The construct pTMV 15-17, kindly provided by Dr. R. S. Nelson, was similar to the infectious construct p35STMVR (discussed in Chapter 3). However, *in vitro* mutagenesis had been applied in order to introduce a mutation within the coat protein ORF. This was located 40 nucleotides downstream from the ATG start codon. As a result a *Bst*X I site and a *Sst* II site were introduced, both were centred at nucleotide 5756 of the TMV-U1 genome. The construct pNSLUR was digested using *Nco* I and *Pst* I. The fragment containing the luciferase gene, TMV 3' untranslated region and ribozyme sequence was purified. It was then ligated into similarly digested pSYN-LUC resulting in a construct designated pBstLU (Figure 5.7). Using the restriction enzymes *Bst*X I and *Pst* I the new insert was excised from pBstLU. Following purification it was then ligated into similarly digested pTMV 15-17, replacing the 3' end of the TMV-U1 genome. This new replication-marker construct was designated pTMVB-LUR (Figure 5.7). It contained a copy of the TMV genome with most of the coat protein gene replaced by the luciferase gene. The luciferase gene start codon was located downstream of the coat protein start codon. In between were the first 42 nucleotides from the TMV-U1 coat protein ORF. These were followed by 39 nucleotides from pSYN-LUC and included two in frame stop codons. The stop codons were situated, in tandem, 15-10 nucleotides upstream from the luciferase gene start codon.

5.2.3 Confirming the Identity of the Replication-Marker Constructs

The identity of pTMVLUR and pTMVB-LUR was confirmed using restriction enzyme digests and Southern blot analysis. DNA fragments were probed with a 679 bp *Sst* II, *Bam*H I fragment isolated p35STMVR (nucleotides 2653-3332 of the TMV-U1 genome). The results, presented in Figure 5.8, suggested that each construct had been

built correctly. Final analysis was carried out by sequencing each constructs using the primers 5P-1 and M13 -20 Forward. The results confirmed that in both constructs, the luciferase gene was correctly inserted between the TMV-U1 coat protein subgenomic promoter and the 3' untranslated region.

It was attempted to excise both constructs from their plasmid vectors using flanking *Pst* I sites and transfer them into the binary vector pPZP111 (Hajdukiewicz *et al.*, 1994). This was to facilitate *Agrobacterium*-mediated plant transformation. Despite repeated attempts, the cloning procedure was not successfully completed.

5.2.4 Microprojectile Bombardment Experiments

Microprojectile bombardment experiments were carried out to test whether the new replication-marker constructs, pTMVLUR and pTMVB-LUR, were capable of expressing luciferase. Each construct was independently bombarded into 24 *N. tabacum* SR1 tissue samples (~2.25 cm^2). The 35S-GUS construct, pRTL2-GUS (Restrepo *et al.*, 1990), was used as an internal control to determine the success of individual bombardments. Each bombardment consisted of ~1.15 µg of the appropriate replication-marker construct and ~0.25 µg of the internal control. Some of the bombardments also included ~0.25 µg of the infectious construct p35STMVR. The results have been presented in Tables 5.1-5.6 and refer to Experiments 5.1-5.6 respectively. Terms used to describe the results have been defined in Section 4.2.5, paragraph 2.

Almost no background luminescence was detected in unbombarded leaf samples (~2.25 cm^2) which had been detached from the plant for either 1 or 2 days prior to analysis (Table 5.1). The GUS activities, ranging from 52.43-140.73 FU min^{-1}, suggested that the bombardments carried out in Experiments 5.2-5.4 had been successful. Furthermore, it appeared that the GUS activity reached a maximum at 2 days post bombardment and that the enzyme was still active at 3 days post bombardment. With one exception, the luciferase activities observed in Experiments 5.2-5.4 were all higher than the activities observed in the unbombarded leaves (Experiment 5.1). However, the activities at 1 (Experiment 5.2) and 3 (Experiment 5.4) days post bombardment were lower than the average activity of 795 LU, determined for unbombarded leaves in Experiment 4.1. The activities recorded at 2 days post bombardment (Experiment 5.3) were also low, especially when compared with the average activity of 3 933 540 LU following the bombardment of pRTS2-LUC into *N. tabacum* SR1 tissue (Experiment 4.2). The low luciferase activities are reflected by the low relative luciferase activity values. Following the 24

bombardments carried out in Experiments 5.5 and 5.6 only three luciferase activities above zero were recorded. However, these values were not higher than the level of background luminescence expected. The accompanying GUS activities, ranging from 0.25-22.35 Fu min^{-1}, were also low. As a result it must be concluded the bombardments conducted in Experiments 5.5 and 5.6 were unsuccessful. The results have been presented to demonstrate that the luciferase activities observed in Experiments 5.2-5.4 were not significantly higher than background.

5.3 DISCUSSION

The development of a TMV based vector capable of replication-dependent expression of the luciferase gene proved to be harder than first anticipated. As a result of the criticisms made concerning the design of the original replication-marker constructs (described in Chapter 4) two new constructs were generated (Figures 5.6 and 5.7). It was hoped that these constructs, designated pTMVLUR and pTMVB-LUR, would express luciferase when delivered into tissue known to host TMV. Transient microprojectile bombardment experiments were carried out to test the constructs, the results are presented in Tables 5.1-5.6. The construct pRTL2-GUS was used as an internal control to establish the success of individual bombardments. The GUS activities indicated that, the bombardments conducted in Experiments 5.5 and 5.6 had failed to successfully deliver DNA into the target tissue. Experiments 5.2-5.4, which involved the independent bombardment of each construct into 12 *N. tabacum* SR1 target tissue samples, had been successful. However, the luciferase activities (~1 000 LU) presented in Tables 5.2-5.4 were not significantly higher than the activities presented in Tables 5.5 and 5.6. In addition, they were approximately equal to levels of background luminescence detected in unbombarded tissue (Table 4.1). It was decided that the constructs should be analysed further once integrated into the genome of *N. tabacum* SR1 plants. The luciferase expressed by newly generated transgenic plants could then be compared with expression by line L, T and C plants (Section 4.2.6.2; Figure 4.13). These had been transformed independently with either the replicase-marker construct from pBU-S4-L1, or a replication-marker constructs from BU-SLU or pBU-SLLUTR respectively (Sections 4.2.2.3; 4.2.5). Despite repeated attempts, the new replication-marker constructs were not successfully transferred into the binary vector pPZP111. There is evidence which suggests that in the presence of certain promoters TMV-like particles can be produced in *E. coli* cells from full length cDNA clones (Yamaya *et al.*, 1988; Hwang *et al.*, 1994). Such particles may have a toxic effect on the bacterial cells and as a result the success of cloning procedures may be affected. On previous occasions difficulty was experienced when working with large fragments from the infectious construct p35STMVR. Despite

being concerned about the inability to move the new constructs into binary vectors, we had no firm proof that it was due to toxicity.

The TMV genome has been successfully used to express a number of foreign genes (Section 1.8; Porta and Lommonossoff, 1996). The replication-marker constructs, pTMVLUR and pTMVB-LUR, have been compared with other TMV-based expression vectors in order to identify potential design faults. It should be noted that it is not easy to directly compare the different TMV-based expression vectors. This is due to the specific objectives of individual research groups, the use of different marker genes, and variations in data obtained and published. The construct pTMVLUR, in particular, was generated with the aid of many intermediate constructs. Sequencing was used to confirm that the luciferase gene had been introduced downstream of the TMV-U1 subgenomic promoter in pTMVLUR and pTMVB-LUR. It also confirmed that each construct contained the TMV-U1 3' untranslated region and downstream ribozyme sequence from pC-35STMVR. During the cloning procedure, restriction enzymes were used to interrupt important gene sequences. Following completion of the constructs it was confirmed that these sites remained functional. Despite the care taken, it is possible that unidentified errors were introduced during the cloning procedure. Replicase gene function could have been tested by the Northern and Western blot analysis of transgenic tissue samples. In order to test that the luciferase gene ORFs remained viable, they could have been cloned into plasmid vectors downstream from either a T3 or T7 promoter. This would have facilitated *in vitro* transcription of the luciferase genes followed by *in vitro* translation (Section 2.9). Luciferase activity assays could then have been performed on the products.

The replication-marker constructs described in Sections 4.2 and 5.2 were designed to be integrated into the genome of plants known to be hosts of TMV. We are unaware of other reports describing TMV-based expression vectors capable of being transcribed *in planta*. Instead, *in vitro* transcription reactions have been used to generate infectious transcripts, these have been manually inoculated on to the leaves of plants or used to infect protoplasts. For the manual inoculation of plants to succeed, it is essential that infectious transcripts maintained the ability to move throughout an inoculated leaf (Section 1.7.2). Due to the 85 nucleotide deletion within the 30 kDa protein ORF of pTMVLUR it was presumed that cell-to-cell movement of this construct was not possible. However, the actual impact of the deletion was not determined. While the 30 kDa ORF does not appear to be required for viral replication (Meshi *et al.*, 1987), it is possible that the deleted region contained important sequences involved with the regulation of gene expression. It also formed part of the OAS which is thought to be important for efficient encapsidation (Section 1.6.7). The construct pTMVB-LUR

should have contained an intact 30 kDa cell-to-cell movement protein gene, and as a result any important factors situated within the ORF. The protein should have been expressed via subgenomic I_2-RNA (Section 1.6.6). Evidence confirming the construct's ability to express the functional movement protein was not obtained. However, if as expected movement was permitted it should have been possible to test the function of pTMVB-LUR within the inoculated leaves of *N. benthamiana* plants (see Section 3.2.3). Once propagated within *N. benthamiana* it should have also been possible to transfer the construct into the leaves of other plants known to host TMV. It should be noted that unless coat protein was supplied *in trans* RNA would have been unprotected and therefore rapidly degraded. In addition, transcripts would have been incapable of efficient systemic movement (Section 1.7.3). Inoculation experiments have not been described in Section 5.2. If able to move from cell-to-cell, RNA transcribed by pTMVB-LUR should also have been capable of inducing lesions on plants expressing the *N* gene. The *N* gene confers resistance to TMV by eliciting the plant's HR response (Section 1.9.1; 1.9.4). One experiment, involving the microprojectile bombardment of *N. tabacum* Samsun *NN* tissue with pTMVB-LUR, has been described in Section 7.2.3.5. The result was not encouraging and the experiment was not repeated.

There was no reason to suspect that the TMV-U1 3' untranslated region was not functioning correctly. Both constructs pTMVLUR and pTMVB-LUR should have contained the complete 3' untranslated region together with 88 nucleotides from the 3' end of the coat protein ORF. Evidence suggests that the sequence required for recognition by the TMV replicase complex does not extend from the untranslated region into the coat protein ORF (Section 1.6.4). Furthermore, Dawson *et al.* (1988) demonstrated that the 3' terminal 175 nucleotides were sufficient to allow replication. The ability of the ribozyme sequence from pC-35STMVR to function has been demonstrated (Dagless *et al.*, 1997). Using local lesion assays on *C. amaranticolor* it was shown that the construct, p35SU1AR, which contained the ribozyme in the reverse orientation was only ~7 % as infectious as p35STMVR.

Due to the deletion present within the 30 kDa protein ORF of pTMVLUR, only 252 TMV-U1 nucleotides remained upstream of the luciferase gene start codon. Evidence suggests that these nucleotides contained the complete coat protein subgenomic promoter (Dawson *et al.*, 1989; Dawson and Lehto 1990). Care was taken to maintain the authentic 3' end of the subgenomic promoter. As a result the region should have been recognisable to the TMV replicase complex (Section 1.6.6). pTMVLUR, contained three additional non-viral nucleotides between the coat protein subgenomic promoter and the luciferase gene start codon. Marsh *et al.* (1988)

suggested that the A/U rich region immediately upstream from the start codon may be required to initiate subgenomic RNA synthesis at the correct site. It has since been suggested that the introduction of foreign nucleotides immediately upstream from the start codon may result in initiation at an alternative site (Lehto *et al.*, 1990). The 3' end of the subgenomic promoter sequence also formed part of the TMV-U1 coat protein translational leader sequence. The three foreign nucleotides, introduced downstream of the authentic 9 nucleotide TMV-U1 leader sequence (Guilley *et al.*, 1979), altered context of the ATG start codon. The importance of maintaining the authentic start codon context remains unclear and has been discussed in Section 4.3. The context of the luciferase gene start codon within pTMVLUR conformed to the optimum models proposed by both Kozak (1984) and Lütcke *et al.* (1987). However the start codon contexts of plant virus genes expressed from subgenomic RNAs do not tend to conform to these models (Section 4.3; Dawson and Lehto, 1990). We remain unsure of the functional significance of the 3 additional nucleotides between the LMC-RNA subgenomic promoter luciferase gene start codon. Other TMV-based expression vectors containing foreign sequence in the same position have been capable of expressing a marker gene (Donson *et al.*, 1991; Kumagai *et al.*, 1993; Chen *et al.*, 1996). Chen *et al.* (1996) replaced the coat protein gene, from a cDNA clone of TMV-U1, with a GFP gene. This was done by inactivating the coat protein start codon (nucleotides 5712-5714) and replacing nucleotides 5721-6147 with the foreign gene. Transcripts produced *in vitro* by the expression vector produced strong fluorescence when manually inoculated on to the leaves of *N. tabacum* Bright Yellow.

The construct pTMVB-LUR contained the authentic TMV-U1 sequence upstream from nucleotide 5753 of the TMV-U1 genome. This included the coat protein subgenomic promoter, the coat protein start codon and a further 39 nucleotides from the 5' end of the coat protein ORF. Consequently the region should have been recognisable to the replicase proteins and capable of directing subgenomic RNA synthesis at the authentic LMC-RNA site (Guilley *et al.*, 1979; Marsh *et al.*, 1988). The exact TMV subgenomic promoter sequences have not yet been defined (Dawson and Lehto 1990). Studies of BMV suggest that the coat protein subgenomic promoter ended immediately upstream of the coat protein start codon (Marsh *et al.*, 1988). However, it is possible that the 39 nucleotides downstream of the coat protein start codon in pTMVB-LUR served some useful purpose. These were followed by 39 nucleotides from pSYN-LUC and then the luciferase gene start codon. Although the start codon remained in frame with the coat protein start codon two stop codons were introduced within the pSYN-LUC sequence. As a result, in order for the luciferase gene to be expressed the ribosomes would be required to reinitiate translation at an internal site. There is evidence to suggest that this is possible although the

efficiency of translation is reduced. Similar expression vectors have been generated where the coat protein gene of either BMV or TMV was replaced by the CAT gene (French e*t al.*, 1986 and Takamatsu *et al.*, 1987 respectively). *In vitro* transcribed RNA proved to be less capable of producing the subgenomic RNAs, compared with the wild type transcripts. Both the described vectors were capable of expressing the CAT gene. Expression was significantly higher if the CAT gene was inserted inframe with the coat protein start codon, compared with vectors where internal initiation was required (Section 1.8). The maximum sequence separating the coat protein gene and CAT gene start codons in the described vectors was 36 nucleotides. This is much shorter that the 81 nucleotide sequence present in pTMVB-LUR.

While the design of the replication-marker constructs is important, the suitability of the luciferase marker gene also requires consideration. Luciferase enzyme activity can be measured by a simple, yet sensitive, assay (Sections 2.13.3; 4.2.1). These assays can be affected by incorrect pH, the presence of heavy metals, and high concentrations of pyrophosphates and salts (DeLuca and McElroy, 1978). The protein is also very unstable and activity is significantly reduced if tissue samples are stored for as little as two hours (personal observation). It has already been observed that RNA would have remained uncoated and as a result unstable. However, similar unprotected RNA has been shown to express high levels of marker proteins (Takamastsu *et al.*, 1987; Chen, 1996). The combined result of producing unstable RNA and from it translating an unstable protein may help explain the low levels of luciferase activity. We are aware of only one report describing the expression of luciferase by a virus-based vector (Joshi *et al.*, 1990). Replication dependent expression of the protein was observed when the gene was used to replace an ORF downstream from the coat protein ORF of barley stripe mosaic virus (BSMV). No expression was observed when the luciferase gene was used to replaced the coat protein ORF, situated at the 5' end of the BSMV RNA "β". It was concluded that RNA transcribed by the second vector was incapable of replication (Joshi *et al.*, 1990). A high error rate has been associated with viral RNA replication due to the absence of proof reading mechanisms. As a result strong selection pressures appear to be responsible for preserving sequences within viral genomes. Such pressures would not apply to foreign genes. The frequency of errors and their impact on the expression of foreign genes, by virus-based vectors, has been discussed by van Vloten-Doting *et al.* (1985) and Siegel (1985). Studies have since suggested that the mutation rate of foreign sequences is not as high as first anticipated (Kearney *et al.*, 1993). The majority of reports concerning successful TMV-based expression vectors describe the use of marker genes which are <1 kb (Section 1.8). The luciferase gene used in the construction of the replication-marker constructs was however ~1.7 kb. If marker genes are unstable or

prone to lethal errors the risk is likely to be higher for larger genes. Donson *et al.* (1991) reported that a TMV-based vector expressing the DHFR gene (238 bp) was more stable than a vector expressing the NPT II gene (832 kb). A second problem may arise due to the size of the luciferase marker gene. This involves the proximity of the start codon to the 3' end of the vector (Section 1.6.8). It has been observed that protein expression, via subgenomic RNA, can be highest from genes positioned closest to the 3' end of the viral genome (French and Ahlquist, 1988; Culver *et al.*, 1993). Culver *et al.* (1993) demonstrated that expression of the 30 kDa protein was proportional to the number of nucleotides deleted downstream from the gene. The start codon of the luciferase gene in the described replication-marker constructs was positioned ~2 kb from the 3' end of the vector. This is more distant than the position of the 30 kDa protein start codon (nucleotide 4903 of the TMV-U1 genome) in relation to the 3' end of the TMV genome (Goelet *et al.*, 1982). It is therefore possible that size of the luciferase gene inhibited its expression. However, constructs described by Donson *et al.* (1991) and Kumagai *et al.* (1993) successfully expressed genes ~800 bp positioned between the TMV-U1 30 kDa protein and coat protein ORFs. The start codons of these genes was positioned ~1.7 kb upstream from the 3' end of the vectors. Furthermore, Lapidot *et al.* (1993) reported detecting GUS activity when the ~1.8 kDa GUS gene was used to replace the TMV-U1 30 kDa ORF.

Analysis of the new replication-marker constructs, pTMVLUR and pTMVB-LUR, is not yet complete. However, the results of microprojectile bombardment experiments, presented in Tables 5.1-5.4 were not encouraging. The discussions presented in this Section and Section 4.3 have considered the design of 4 different replication-marker constructs. As a result possible design faults have been identified. It has been decided that the generation and analysis of *N. tabacum* SR1 plants independently transformed with pTMVLUR and pTMVB-LUR is required. This would complement the further analysis of the transgenic plants described in Chapter 4. The results obtained from the analysis of DNA, RNA and protein expression may provide important information concerning the function of the constructs. We have recently received similar TMV-based constructs, designed to express either GUS or a GFP (kindly donated by Dr. Curtis Holt, Department of Biology, Washington University, USA). The ability of the construct to function needs to be fully be determined (information concerning the design of the construct has not yet been supplied). It may not be wise to design further constructs until information concerning the function of existing constructs has been obtained.

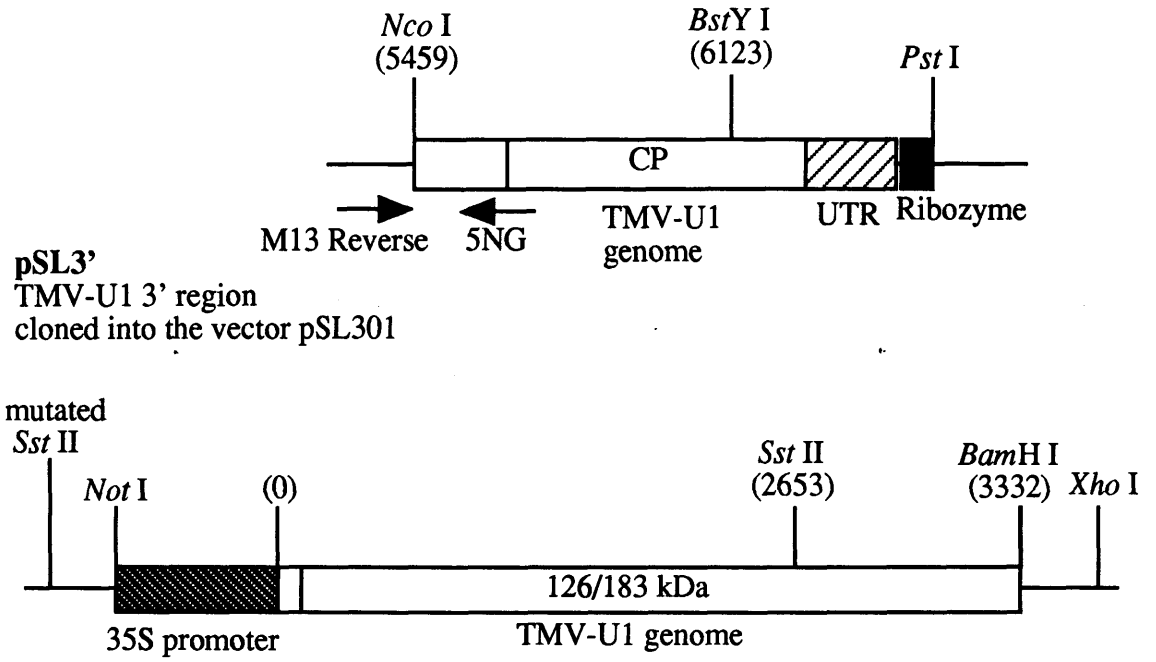


Figure 5.1 Diagrams of pSL3' and p5'TMV. Restriction enzyme sites and primer binding sites used during construction have been indicated. Numbers in brackets refer to nucleotides of the TM-U1 genome.

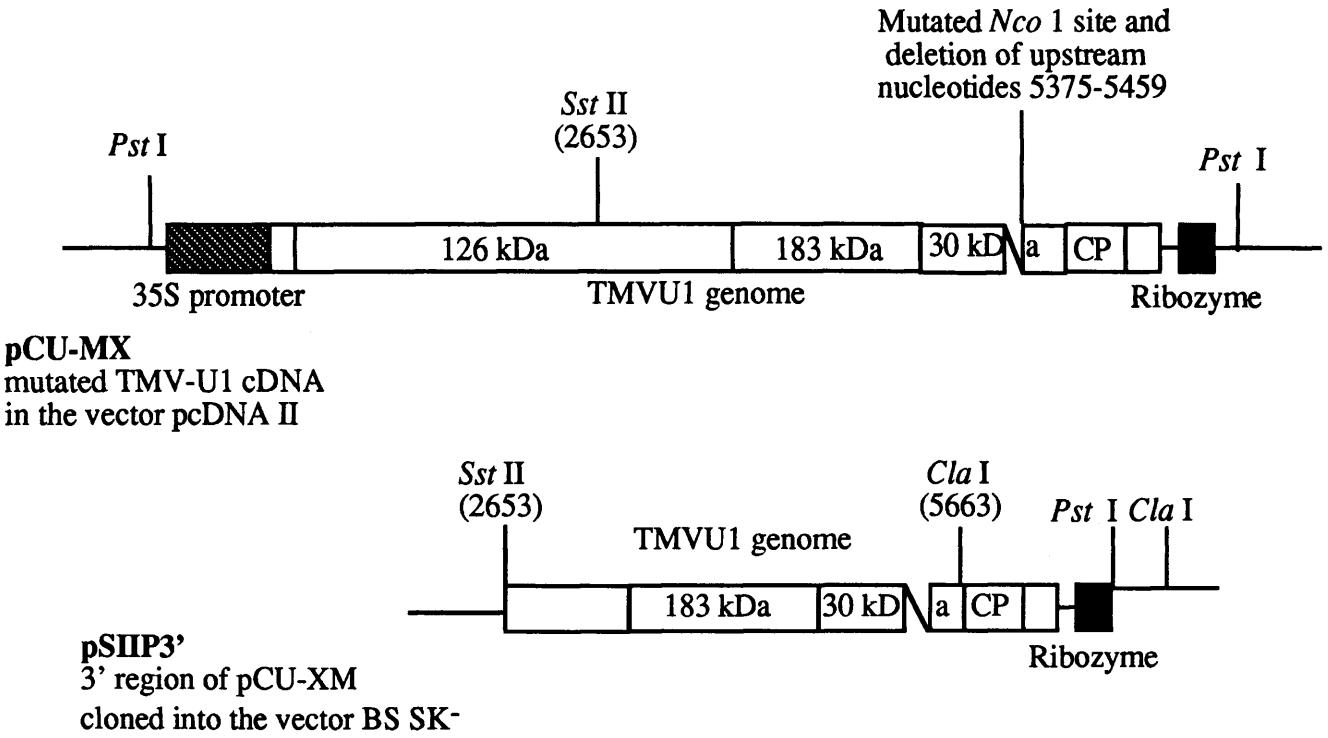
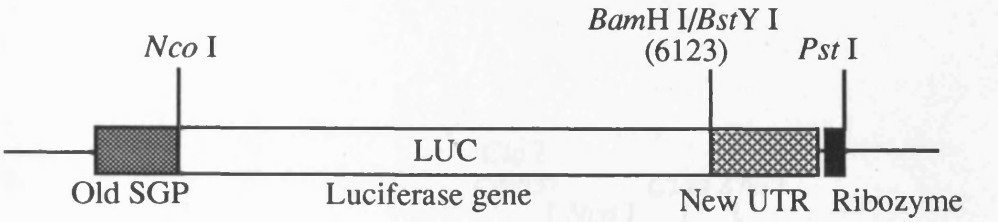
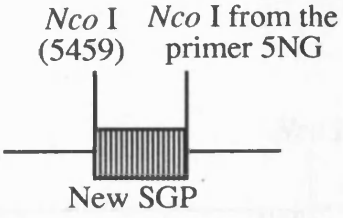


Figure 5. 2 Diagrams of pCU-XM and pSIIP3'.

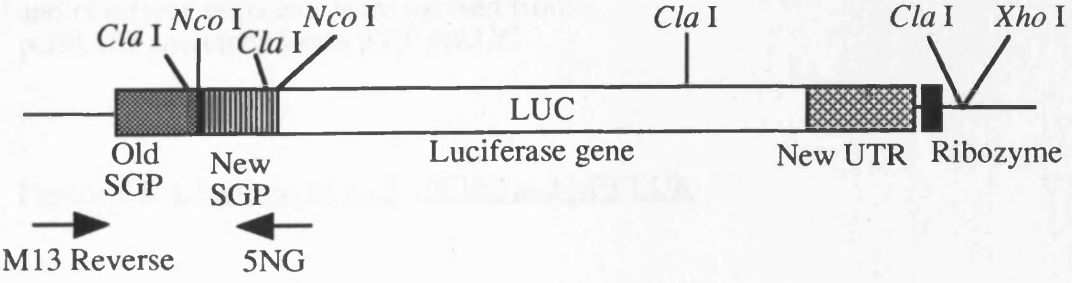


pNSLUR
A new 3' untranslated region and ribozyme sequence was cloned into the vector pS4-L1

Figure 5.3 Diagram of pNSLUR. The primer binding site used for sequencing has been indicated.

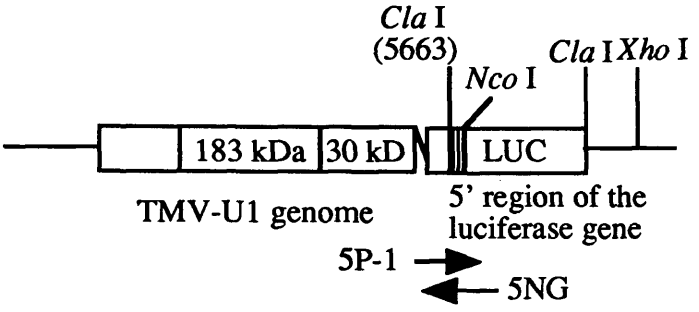


pGSGP
A new subgenomic promoter was amplified from pSL3' and cloned into the vector pGEM T

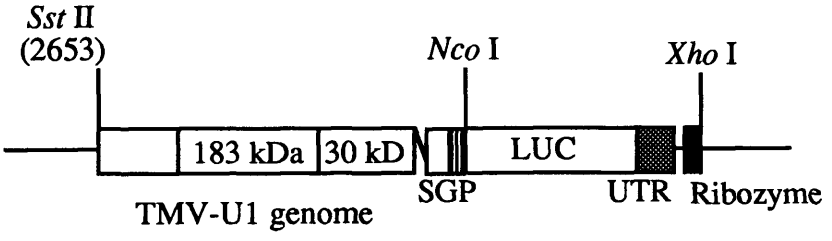


pGSLUR
The new subgenomic promoter was excised from pGSGP and cloned into the vector pNSLUR

Figure 5.4 Diagrams of pGSGP and pGSLUR. Primer binding sites used for PCR amplification and sequencing have been indicated.



pT3'1/2LUC
The new SGP, together with the 5' region of the luciferase gene was excised from pGSLUR and cloned into pSIIP3'



pT3'LUR
The luciferase gene, 3' untranslated region and ribozyme sequence were excised from pGSLUR and cloned into pT3'1/2LUC

Figure 5.5 Diagrams of pT3'1/2LUC and pT3'LUR.

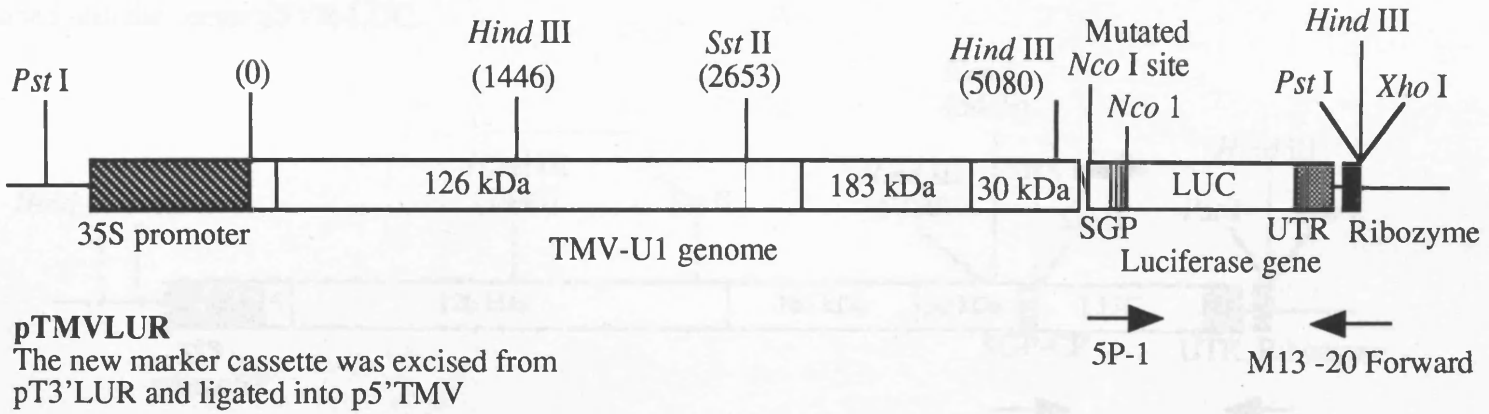


Figure 5.6 Diagram of the New Replication-marker Construct pTMVLUR. The completed constuct was harboured in the plasmid vector pBSXSII. Restriction enzyme sites used for diagnosis (see Figure 5.8) as well as cloning have been indicated, together with primer binding sites used for sequencing.

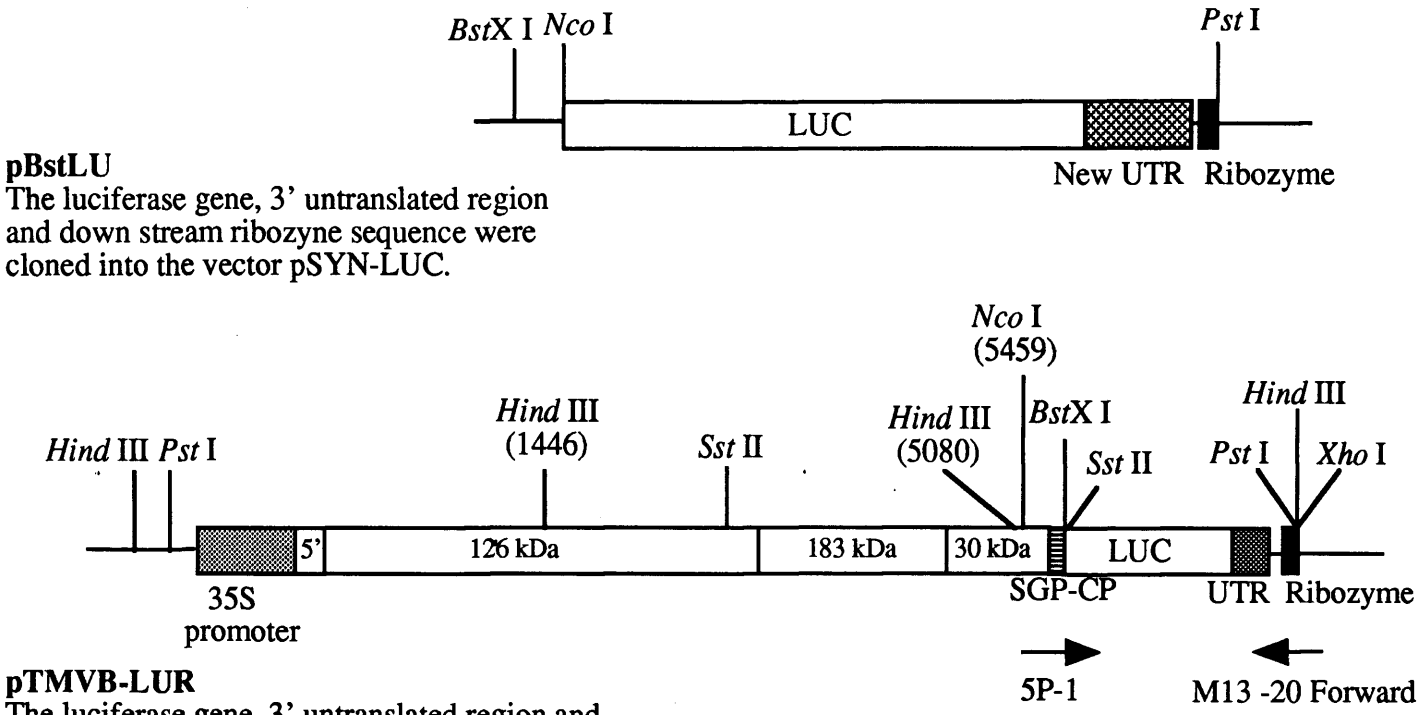
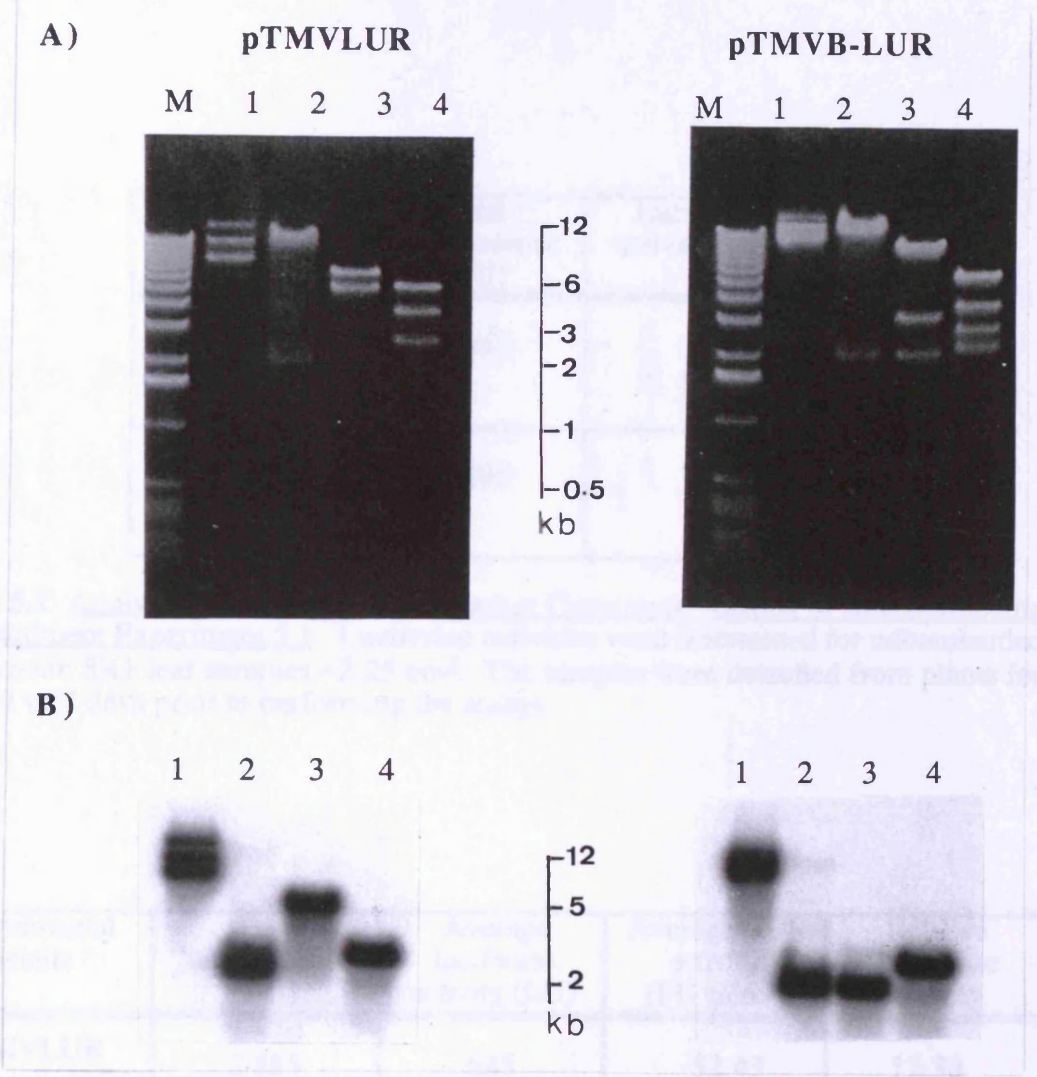


Figure 5.7 Diagrams of pBstLU and the New Replication-marker Construct pTMVB-LUR. The completed construct, pTMVB-LUR, was harboured in the plasmid vector pAlter-1. Restriction enzyme sites used for both cloning and diagnosis (see Figure 5.8) have been indicated, together with primer binding sites used for sequencing.



C)

Lane	Enzymes	Expected fragment size following digestion of pTMVLUR	Expected fragment size following digestion of pTMVB-LUR
2	*Nco* I, *Xho* I	9.1 kb, 2.2* kb	11.9 kb, 2.0* kb, 0.3 kb
3	*Sst* II, *Xho* I	6.3 kb, 5.0* kb	9.1 kb, 3.1 kb, 2.0* kb
4	*Hind* III	5.1 kb, 3.6 kb, 2.6* kb	5.7 kb, 3.6 kb, 2.7* kb 2.2 kb

Figure 5.8 Restriction Enzyme Digests of pTMVLUR and pTMVB-LUR. DNA fragments were visualised on agarose gels (A). Molecular weight markers (M), undigested DNA (lanes 1). Southern blots of (A) probed with an ~750 bp *Eco*R I, *Eco*R V luciferase gene fragment isolated from pRTS2-LUC (B). The blots were exposed to X-ray film for 4 hours. Table showing restriction exzymes used and expected band sizes (C), * indicates the fragments to which the probe hybridised.

Experimental details	Total luminescence (LU)	Luciferase activity (LU)
Unbombarded sample, detached for 1 day	2 445	5
Unbombarded sample, detached for 2 days	1 495	0

Table 5.1 Analysis of the Replication-marker Constructs: Results of Microprojectile Bombardment Experiment 5.1. Luciferase activities were determined for unbombarded *N. tabacum* SR1 leaf samples ~2.25 cm^2. The samples were detached from plants for either 1 or 2 days prior to performing the assays.

Experimental details	Total luminescence (LU)	Average luciferase activity (LU)	Average GUS activity (FU min^{-1})	Relative luciferase activity
pTMVLUR assayed at 1 dpb.	2 820 2 585 4 630	645	52.43	12.30
pTMVB-LUR assayed at 1 dpb.	2 965 4 530 2 805	0	62.83	0

Table 5.2 Analysis of the Replication-marker Constructs: Results of Microprojectile Bombardment Experiment 5.2. *N. tabacum* SR1 leaf samples ~2.25 cm^2 were assayed at 1 day post bombardment with either pTMVLUR or pTMVB-LUR. The construct pRTL2-GUS was included as an internal control to determine the success of bombardments.

Experimental details	Total luminescence (LU)	Average luciferase activity (LU)	Average GUS activity (FU min^{-1})	Relative luciferase activity
pTMVLUR assayed at 2 dpb.	4 395 3 230 4 535	1 083	140.73	7.70
pTMVB-LUR assayed at 2 dpb.	6 085 3 370 4 205	1 083	117.97	9.18

Table 5.3 Analysis of the Replication-marker Constructs: Results of Microprojectile Bombardment Experiment 5.3. *N. tabacum* SR1 leaf samples ~2.25 cm^2 were assayed at 2 days post bombardment with either pTMVLUR or pTMVB-LUR. The construct pRTL2-GUS was included as an internal control to determine the success of bombardments.

Experimental details	Total luminescence (LU)	Average luciferase activity (LU)	Average GUS activity (FU min^{-1})	Relative luciferase activity
pTMVLUR assayed at 3 dpb.	4 005 6 510 2 390 3 800 3 955 2 945	704	74.27	9.48
pTMVB-LUR assayed at 3 dpb.	3 665 4 360 6 950 3 980 4 470 5 995	273	90.87	3.00

Table 5.4 Analysis of the Replication-marker Constructs: Results of Microprojectile Bombardment Experiment 5.4. *N. tabacum* SR1 leaf samples ~2.25 cm^2 were assayed at 3 days post bombardment with either pTMVLUR or pTMVB-LUR. The construct pRTL2-GUS was included as an internal control to determine the success of bombardments.

Experimental details	Total luminescence (LU)	Luciferase activity (LU)	GUS activity (FU min^{-1})	Relative luciferase activity
pTMVLUR young leaf sample assayed at 2 dpb.	1 715	215	1.10	195.45
pTMVLUR mature leaf sample assayed at 2 dpb.	2 260	0	0.50	0
pTMVB-LUR young leaf sample assayed at 2 dpb.	1 820	0	7.45	0
pTMVB-LUR mature leaf sample assayed at 2 dpb.	1 640	0	0.90	0
pTMVLUR and pC-35STMVR young leaf sample assayed at 2 dpb.	2 495	0	8.80	0
pTMVLUR and pC-35STMVR mature leaf sample assayed at 2 dpb.	2 320	0	3.80	0
pTMVB-LUR and pC-35STMVR young leaf sample assayed at 2 dpb.	2 490	90	0.25	360.00
pTMVB-LUR and pC-35STMVR mature leaf sample assayed at 2 dpb.	1 540	0	23.35	0

Table 5.5 Analysis of the Replication-marker Constructs: Results of Microprojectile Bombardment Experiment 5.5. Either young or mature *N. tabacum* SR1 leaf samples ~2.25 cm^2 were assayed at 2 days post bombardment with either pTMVLUR or pTMVB-LUR. In some cases the constructs were co-bombarded with the infectious construct pC-35STMVR. The construct pRTL2-GUS was included as an internal control to determine the success of bombardments.

Experimental details	Total luminescence (LU)	Average luciferase activity (LU)	Average GUS activity (FU min^{-1})	Relative luciferase activity
pTMVLUR young leaf sample assayed at 3 dpb.	1 740 2 170	0	10.57	0
pTMVLUR mature leaf sample assayed at 3 dpb.	1 535 1 650	0	2.47	0
pTMVB-LUR young leaf sample assayed at 3 dpb.	1 395 2 055	0	6.70	0
pTMVB-LUR mature leaf sample assayed at 3 dpb.	2 355 2 135	0	2.00	0
pTMVLUR and pC-35STMVR young leaf sample assayed at 3 dpb.	2 655 2 495	0	22.35	0
pTMVLUR and pC-35STMVR mature leaf sample assayed at 3 dpb.	1 745 1 670	0	2.75	0
pTMVB-LUR and pC-35STMVR young leaf sample assayed at 3 dpb.	2 595 2 075	35	2.65	13.21
pTMVB-LUR and pC-35STMVR mature leaf sample assayed at 3 dpb.	1 255 1 785	0	0.97	0

Table 5.6 Analysis of the Replication-marker Constructs: Results of Microprojectile Bombardment Experiment 5.6. Either young or mature *N. tabacum* SR1 leaf samples ~2.25 cm^2 were assayed at 3 days post bombardment with either pTMVLUR or pTMVB-LUR. In some cases the constructs were co-bombarded with the infectious construct pC-35STMVR. The construct pRTL2-GUS was included as an internal control to determine the success of bombardments.

CHAPTER 6

THE TRANSFORMATION OF *L. ESCULENTUM* AND *N. TABACUM* SR1 WITH TMV-BASED REPLICATION AND REPLICASE CONSTRUCTS

6.1 INTRODUCTION

The ability of the *Tm-1* gene to confer resistance to TMV has been well documented (Section 1.9.2). The resistance has been shown to be effective in intact *L. esculentum* plants (Pelham, 1972; Fraser and Loughlin, 1980) and also in leaf mesophyll cell protoplasts isolated from such plants (Motoyoshi and Oshima, 1977; Motoyoshi and Oshima, 1979 Watanabe *et al.*, 1987). Although the gene demonstrates dominance by suppressing symptom formation in the heterozygous state, its ability to reduce virus multiplication is only partially dominant. Even in the heterozygous state a significant reduction in multiplication of 70-75 %, compared with 90-95 % in the homozygous state, has been observed (Fraser and Loughlin, 1980). Many investigations involving the *Tm-1* gene have been carried out using tomato strains of TMV (Section 1.2) as these cause the greatest problem to field grown tomatoes (Ohno *et al.*, 1984). However, the gene is effective against many strains of the virus, but not always to the same extent. For example, TMV-U1 and TMV-OM, unlike TMV-L, are not associated with resistance breaking isolates (Meshi *et al.*, 1988). Furthermore, unlike some of the Ohio isolates (Fraser and Loughlin, 1980), TMV-U1 does not appear to demonstrate temperature sensitivity (personal observation). It therefore fails to multiply in host plants expressing the *Tm-1* gene, even when plants are grown at high temperatures. The way in which the *Tm-1* gene interacts with TMV is not understood. However, Meshi *et al.* (1988) demonstrated the TMV encoded replicase proteins (Section 1.7.1) may interact with the *Tm-1* gene to confer the resistance (Section 1.9.2).

It was decided to investigate the function of the *Tm-1* gene and its possible interaction with TMV encoded replicase proteins further. This was to be achieved by comparing levels of TMV RNA and protein following the stable transformation of i) non-resistant *L. esculentum* GCR 26 (+/+); ii) near isogenic *L. esculentum* GCR 237 (*Tm-1/Tm-1*) plants, homozygous for the *Tm-1* resistance gene. Two suitable 35S-constructs were chosen for the transformation experiments. The first, to be known as the "replication construct", consisted of almost the entire TMV-U1 genome and as a result transcribed RNA should have been capable of replication and the expression of subgenomic RNAs. However, a deletion in the 30 kDa ORF meant that RNA would have been incapable of moving from cell-to-cell (Section 1.7.2) or assembly into virus particles (Section 1.6.7). The inability of RNA to move between cells was considered to be an advantage. It would enable transgenic plants to be studied throughout their entire life cycle without concerns regarding the escape of genes encoding the infectious disease. The second construct, to be known as the "replicase construct", was designed to efficiently express the TMV-U1 126 kDa and 183 kDa

replicases proteins. However, removal of the 3' end of the genome ensured that RNA, within transformed cells, would have been incapable of replication and the expression of subgenomic RNAs (Sections 1.5.1; 1.6.4).

6.2 RESULTS

6.2.1 Construct Preparation

A movement deficient cDNA clone of TMV-U1, designated pCU-XM, has been described in Section 5.2.1.2 and suited our need of a replication construct. The 35S promoter allowed viral RNA to be transcribed *in planta* and a ribozyme sequence, inserted downstream from the 3' end of the TMV genome, ensured that additional non-viral nucleotides were limited. Sequence analysis revealed that it lacked nucleotides 5375-5459 of the TMV-U1 genome. The 35S-cDNA construct needed to be transferred from pcDNA II into a binary vector prior to plant transformation. The construct was excised from pcDNA II using flanking *Pst* I sites and religated into the *Pst* I digested, dephosphorylated binary vector pPZP111 (Hajdukiewicz *et al.*, 1994). Like pBin 19 the T-DNA region of pPZP111 contained the kanamycin resistance gene, enabling the selection of transformed plant lines. The identity of the new binary construct, designated pBU-XM (Figure 6.1), was confirmed by restriction enzyme digests and Southern blot analysis (Figure 6.3). The results enabled the construct to be orientated within pPZP111 and showed that the 5' end of the 35S promoter was closest to the M13 Reverse primer site.

The replicase construct was built and transferred to a binary vector using three cloning steps (Figure 6.2). The infectious construct pC-35STMVR (described in Chapter 3) was used to supply the replicase genes under the control of the 35S promoter. Expression of the genes was enhanced by the TMV 5' untranslated leader sequence (Section 1.6.3). In order to prevent replication the 3' end of the TMV genome, together with the downstream ribozyme sequence, was replaced by transcription termination and polyadenylation signals from CaMV. The CaMV terminator sequence was removed from the gene expression vector pWP83 (Paul *et al.*, 1995) using *Eco*R I and *Eco*R V. It was then cloned into similarly digested pSL301. This intermediate construct, designated pCaTerm, contained suitably located *Nco* I and *Kpn* I restriction enzyme sites allowing the CaMV terminator sequence to be transferred into *Nco* 1 (nucleotide 5459 of the TMV-U1 genome) and *Kpn* I digested pC-35STMVR. The new replicase construct was designated pTRepTerm. Prior to plant transformation the construct was excised from pcDNA II using *Xho* I and *Kpn* I and transferred into *Sal* I, *Kpn* I digested pBin 19. The identity of the binary replicase

construct, designated pBTRepTerm (Figure 6.2) was confirmed by restriction enzyme digests and Southern blot analysis (Figure 6.3).

6.2.2 Transformation of *L. esculentum.*

The transformation procedure described in Section 2.12.4 was strictly followed in order to transform *L. esculentum* cotyledons with either the replication construct from pBU-XM or the replicase construct from pBTRepTerm. The transformations were carried out using cotyledons prepared from eight day old *L. esculentum* GCR 26 (+/+) and *L. esculentum* GCR 237 (*Tm-1/Tm-1*) seedlings. Each independent transformation required 40 seedlings, resulting in 160 explants. Following co-cultivation with the *Agrobacterim* cultures, the explants were transferred to plates containing fresh selective shoot regeneration medium at weekly intervals, for a period of eight weeks. During this time the majority of explants remained healthy, however, no calli or shoots developed. The explants prepared from *L. esculentum* cotyledons appeared to be more sensitive to wounding than previously described *N. tabacum* SR1 leaf disc explants (Sections 3.2.6; 4.2.5). As a result ~25 % of explants deteriorated over the 8 week incubation period and were discarded. The explants were incubated for a further 4 weeks, still no calli or shoots developed. As expected, slight *Agrobacterium* growth was observed following the co-cultivation of explants with the *Agrobacterium* cultures. However, further growth was inhibited once the explants were transferred to the medium containing augmentin. At no time during the 12 week incubation period were phenolic compounds allowed to contaminate the growth medium.

The reasons behind the failure of the described *L. esculentum* transformations required investigation. As explained at the beginning of Section 2.12.4, the protocol followed for the transformations was received from Dr. A. Pfitzner (Charng and Pfitzner, 1994 and personal communication). It was based on 2 previously described protocols (Filatti *et al.*, 1987; Smith *et al.*, 1988) and had been optimised for the transformation of Craigella and Moneymaker cultivars. We were in possession of a second protocol used by The Sainsbury Laboratory (K. Harrison, John Innes Centre for Plant Science Research, Norwich, UK., personal communication). This had been optimised for the cultivar Moneymaker and was based on the protocol described by Filatti *et al.* (1987) and a protocol described by McCormick *et al.* (1986). A more recent report by Hamza and Chupeau (1993) reconsidered the conditions required for successful transformation of *L. esculentum.* In particular, the contents of shoot regeneration medium was investigated. As a result of studying the different protocols, we decided to place explants isolated from 8 day old *L. esculentum* GCR 26 (+/+) cotyledons on 5 different types of shoot regeneration medium. It was thought that this

may identify optimal media for the regeneration of *L. esculentum* Mill. cv. Craigella GCR lines. Five different types of media were chosen for assessment, their contents are detailed in Table 6.1. The Pfitzner and Harrison shoot regeneration media were described in the two transformation protocols received (personal communications mentioned above). Pfitzner-a and Pfitzner-b were variations of the Pfitzner medium based on recommendations reported by Hamza and Chupeau (1993). MSD4X2 had been successfully used for the regeneration of *N. tabacum* plants (Sections 3.2.6; 4.2.5). Approximately 50 *L. esculentum* GCR 26 (+/+) explants were incubated on each type of shoot regeneration media for 28 days. The explants were then photographed (Figure 6.4) and the number of developing shoots counted (Table 6.2). Both calli and shoots developed following incubation on all five types of media. MSD4X2 was judged to be the least suitable medium, despite extensive callus formation fewer shoots developed. Following visual examination, these shoots were judged to be the least healthy. A similar number of shoots developed as a result of incubating explants on the other 4 types of media. Shoots appeared to be particularly healthy following incubation on either Pfitzner or Harrison shoot regeneration media, some shoots displayed clearly distinguishable *L. esculentum* leaflets. The addition of IAA or reduction of zeatin riboside in Pfitzner medium appeared to increase callus formation, yet this did not lead to increased numbers of shoots.

6.2.3 Transformation of *N. tabacum* SR1

6.2.3.1 Regeneration of Transgenic Plants

It was decided to transform *N. tabacum* SR1 independently with the constructs pBU-XM and pBTRepTerm. If successful, it would confirm that the constructs and the *Agrobacterium* cultures in which they were harboured were suitable for plant transformation. The transformation was carried out according to the protocol described in Section 2.12.3. Following the co-cultivation of *N. tabacum* SR1 leaf discs with the *Agrobacterium* cultures, the growth of callus was observed within 3 weeks. This led to the appearance of shoots by 4 weeks post co-cultivation. Following the development of kanamycin resistant roots, the T_0 plants were transferred to soil and used for preliminary analysis. It was decided that further analysis should be carried out on the T_1 generation. PCR was used in order to determine whether the constructs had been stably integrated into the plant genome. Using primers 5'REP and 3'REP (previously described in Section 3.2.6) it was possible to amplify a 735 bp fragment from the coding region of the TMV replicase proteins (nucleotides 2485-3219 of the TMV-U1 genome).

The transformation of *N. tabacum* SR1 leaf discs with the replication construct from pBU-XM led to the regeneration of 8 plants, these were numbered and given the prefix "X". All the plants (X1-X8) appeared to be healthy and did not display the symptoms associated with wild type TMV infections (Section 1.3). Despite appearing to be healthy the plant X2 developed very slowly and was therefore excluded from analysis. Following PCR amplification and agarose gel electrophoresis, the amplified bands were faint and have not photographed well (Figure 6.5). DNA was therefore transferred to a membrane and probed with an ~700 bp *Sst* II, *Bam*H I fragment isolated from pC-35STMVR (nucleotides 2653-3332 of the TMV-U1 genome). The result suggested that the replication construct had been stably integrated into the genomes of plant lines X5, X7, and X8 (Figure 6.5). An element of doubt was associated with the negative result obtained for plant lines X1, X3, X4 and X6, as it is unusual to regenerate so many escapes using kanamycin selection. Following the co-cultivation of *N. tabacum* SR1 leaf discs with the replicase construct from pBTRepTerm 17 plants were regenerated. They were numbered and given the prefix "B". All line B plants appeared to be healthy and 8 were chosen for analysis (B1-B8). The result obtained following PCR analysis suggested that plant lines B1, B2, B7 and B8 contain the stably integrated replicase construct (Figure 6.5). Southern blot analysis revealed faint bands (~700 bp) following the PCR amplification of tissue from plants B3, B4, B5 and B6. Further analysis of these lines was required, especially as a faint band was also observed following Southern blot analysis of an untransformed *N. tabacum* SR1 sample.

6.2.3.2 Western Immunoblot Analysis

Protein samples were prepared using tissue from the T_0 plants X1, X3-X8 and B1-B8, using protein extraction buffer B (Section 2.13.2.2). Approximately equal concentrations of protein were used for SDS-PAGE, protein was then transferred to membranes and probed with antibody raised to the 126 kDa TMV encoded replicase protein. Visualisation was carried out using an alkaline phosphatase conjugated secondary antibody. The 126 kDa replicase protein was not detected in samples prepared from line X or B plants following development for 30 minutes (Figure 6.6). Faint bands in the region of the protein were observed. It was thought that these were due to background staining as a similar faint band was visible in a sample prepared from an untransformed *N. tabacum* SR1 plant, following development for only 10 minutes. The 126 kDa replicase protein was clearly visible in a sample prepared from TMV-U1 infected tissue, following development for 10 minutes. The procedure was repeated, using protein samples prepared from line X plants and a more sensitive horseradish peroxidase conjugated secondary antibody. The 126 kDa protein remained

undetected following exposure of the membrane to X-ray film for 10 minutes (result not shown). The protein was clearly visible in a sample prepared from TMV infected *N. tabacum* SR1 tissue. The replication construct from pBU-XM should have been capable of expressing the TMV replicase proteins, replication of RNA, production of subgenomic RNAs and as a result, expression of the TMV coat protein. Western immunoblots of protein sampled from line X plant tissue were therefore probed with antibody raised to the 17.5 kDa TMV-U1 coat protein. Following visualisation, using an alkaline phosphatase conjugated secondary antibody, coat protein was clearly identified in samples prepared from plants X5 and X8 (Figure 6.7). The results of PCR analysis suggested that both these lines had been successfully transformed with the replication construct (Section 6.2.3.1). Faint bands (~17 kDa) were also visible in samples prepared from the plants X4, X6 and X7. The level of coat protein detected, even in tissue sampled from plant X5, was below that detected in TMV infected *N. tabacum* SR1 tissue (Figure 6.7). No coat protein was detected in a sample prepared from an untransformed, healthy *N. tabacum* SR1 plant.

6.2.3.3 Infection of Transgenic Plants with TMV

Reports that transgenic plants expressing part of the TMV genome exhibit varying degrees of resistance to TMV are discussed in Section 1.9.6. It was decided to carry out a preliminary investigation, to determine whether any of the transgenic line X or line B *N. tabacum* SR1 plants demonstrated resistance to TMV. As the plants were required to produce seed (to allow further analysis of the T_1 generation) the investigation involved inoculating detached leaves. Prior to inoculation it was confirmed, using RNA dot blots probed with an ~3.6 kb *Hind* III fragment isolated from pC-35STMVR, that the regenerated plants did not contain detectable TMV RNA (result not shown). Leaves from 7 plants were chosen, these were X3, X4, X6, B1, B2, B6 and B8. PCR analysis suggested that the regenerated plants B1, B2, and B8 contained stably integrated copies of the replicase construct from pBTRepTerm (Figure 6.5). However, it had failed to confirm the transgenic nature of plants X3, X4, X6 and B6. Leaves from these plant had been chosen due to a shortage of suitable material. Each leaf was inoculated with ~5 µg of purified TMV-U1 particles, at the same time two control leaves detached from untransformed healthy *N. tabacum* SR1 plants were inoculated. No symptoms developed over a 5 day incubation period and the leaves appeared to remain healthy, despite being detached from their respective plants. At 5 days post inoculation RNA dot blots were again used to determine whether a TMV infection had spread throughout the leaves (Figure 6.8). TMV RNA was detectable in both samples prepared from untransformed *N. tabacum* SR1 leaves. However, the level of RNA appeared to be much higher in one of the samples. TMV

RNA was detectable in the sample taken from the detached leaf of plant X4. However, it was undetectable in samples prepared from the detached leaves of plants X3, X6, B1, B2, B6 and B8. While the absence of detectable TMV RNA could indicate resistance, it is also possible that the inoculation procedure had failed.

6.3 DISCUSSION

Due to failure of the attempted *L. esculentum* transformations, the results presented in this Chapter differ from the objectives set out in the introduction. These were to use plant transformation in order to investigate possible interactions between the TMV encoded replicase proteins and the *Tm-1* resistance gene. Two 35S-TMV cDNA constructs were built in binary vectors. It was thought that RNA transcribed from the replication construct, pBU-XM, should replicate and produce viral coat protein, but not move from cell-to-cell or assemble into virus particles. This was due to the deletion of TMV-U1 nucleotides 5375-5459 (Section 5.2.1.2) which correspond to amino acids 158-187 of the 30 kDa protein. The deleted amino acids are thought to be involved both with binding single-stranded RNA (Citovsky *et al.*, 1992) and with increasing the plasmadesmata size exclusion limit (Waigmen *et al.*, 1994), both functions being essential for cell-to-cell movement. The deletion also removed part of the OAS and included nucleotides thought to be essential for the assembly of RNA into virus particles (Turner *et al.*, 1988). The replicase construct, pBTRepTerm, was designed to express high levels of the TMV replicase proteins. Although the majority of protein was expected to be the 126 kDa protein, readthrough of the amber stop codon at TMV nucleotide 3537 meant that 5-10 % of protein should have been the larger 183 kDa replicase protein (Pelham, 1978). The absence of TMV-U1 nucleotides 5464-6395, which include the 3' untranslated region, meant that RNA transcribed from the replicase construct should have been incapable of replication (Miller *et al.*, 1986; Ishikawa *et al.*, 1988 Takamatsu *et al.*, 1990b).

Attempts were made to transform the constructs independently into non-resistant *L. esculentum* GCR 26 (+/+) and near isogenic, TMV resistant *L. esculentum* GCR 237 (*Tm-1/Tm-1*) plants. Although this did not result in the development of shoot or even callus, the majority of explants remained healthy for over 8 weeks. Southern blot analysis was used in order to determine that the constructs had been assembled in binary vectors and conjugated into *A. tumefaciens* strain LBA4404. It was still possible that the constructs had not been correctly cloned into a functional T-DNA region. This was however, unlikely to be the case for both the replication and the replicase constructs, especially as two different binary vectors, pPZP111 and pBin 19 had been used. It was also possible that the *vir* gene, essential for the binary

vector system of gene transfer, was not correctly functioning. A further point to note is that, when working with constructs containing genes from plant pathogens, there is a risk that the constructs may prove to be toxic to cells. The low number of plants regenerated following transformation with the infectious construct from pB-35STMV has already been commented on (Section 3.3). Fears over the suitability of the binary replication and replicase constructs were allayed following the successful transformation of *N. tabacum* SR1 plants. However, non-lethal effects induced by the constructs could still have contributed to the failure of *L. esculentum* transformations.

There are many cultivars of *L. esculentum* Mill., these include Craigella, Moneymaker, UC82B, Alisa Craig, Beefmaster, Bigboy, Betterboy and Perfect Peel. Many of the lines produced from these cultivars possess favourable characteristics which suit them both to crop production and research. In order to study the *Tm-1* gene we were limited to near isogenic lines from a Craigella GCR cultivar. It was not anticipated that transformation would prove to be a problem as the method described by Dr. A. Pfitzner had been optimised for the transformation of Craigella and Moneymaker cultivars. It was decided to establish whether the shoot regeneration medium was suitable for the regeneration of *L. esculentum* GCR 26 (+/+) plants. At the same time incubation of explants on several different types of media (Table 6.1) helped determine whether the contents of the media could have been further optimised. The results presented in Figure 6.4 and Table 6.2 demonstrate the suitability of the Pfitzner medium, although comparable results were obtained using Harrison medium. The appearance of distinguishable *L. esculentum* leaflets following incubation on Pfitzner and Harrison media was important, as shoots with abnormal morphology have in some cases been reported (Hamza and Chupeau, 1993). Hamza and Chupeau, (1993) reported that supplementing endogenous levels of the auxin, IAA, could improve the regeneration efficiency. However the incubation of explants on Pfitzner-a medium which contained IAA had no positive effect. All the media tested with the exception of MSD4X2 contained zeatin riboside. Zeatin is a naturally occurring cytokinin which interacts with auxin in order to stimulate cell division and differentiation. Although Hamza and Chupeau, (1993) accepted the benefit of zeatin riboside in shoot regeneration medium they suggested that the concentration could be reduced from 2 mg lt^{-1} to 0.5 mg lt^{-1}. No positive effects were observed following the incubation of explants on Pfitzner-b medium which contained only 0.5 mg lt^{-1} zeatin riboside. MSD4X2 is a medium widely used with in the Department of Botany (University of Leicester) for the regeneration of *N. tabacum* plants. The ability of *L. esculentum* to regenerate on MSD4X2 was tested without evidence suggesting its suitability. It was not surprising that the medium proved to be the least suitable.

Having determined the suitability of the shoot regeneration medium other aspects of the transformation procedure have been considered. Both *A. tumefaciens* strain LBA4404 and the binary vector pBin 19 had been used by Dr. A. Pfitzner to successfully transform *L. esculentum*. Although we were unaware whether the binary plasmid pPZP111 had been used to transform *L. esculentum*, it had been used to successfully transform *N. tabacum* (Hajdukiewicz *et al.*, 1994). It was generally considered that 8 day old cotyledons produced explants most suited to the transformation and regeneration of *L. esculentum* (Fillatti *et al.*, 1987; Hamzu and Chupeau, 1993; Dr. A. Pfitzner and K. Harrison, personal communications). Furthermore, the co-cultivation of explants with *Agrobacterium* cultures for 48 hours had also been strongly recommended (McCormick *et al.*, 1986; Fillatti *et al.*, 1987; Hamza and Chupeau, 1993; Dr. A. Pfitzner and K. Harrison, personal communications). Two aspects of the procedure followed for the attempted *L. esculentum* transformations differed from advice given in the other protocols studied. Despite detailed instruction on the preparation of the *Agrobacterium* cultures used for co-cultivation, the protocol described by Dr. A. Pfitzner did not recommend specific dilution of the cultures. This conflicts with other protocols. In particular Fillatti *et al.*, (1987) reported the optimal concentration of *A. tumefaciens* strain LBA4404 to be $5x10^8$ bacteria in 1 ml of culture. Perhaps the most important step which we did not include was the preincubation and co-cultivation of cotyledon explant on feeder plates containing a layer of tobacco suspension cells. This was recommended in the majority of protocols studied (Horsch *et al*, 1985; McCormick *et al..*, 1986; Fillatti *et al.*, 1987; Hamza and Chupeau, 1993; K. Harrison, personal communication), although the length of preincubation varied from 8-48 hours. The use of feeder cells was reported to at least double the transformation efficiency despite a slight decrease in the ability of transformed cells to form shoots (Fillatti *et al.*, 1987; Hamza and Chupeau, 1993). It has been reported that the feeder cells may either boost *vir* gene inducing compounds, or help reduce the effects of bacteria induced stress on the cotyledons (Fillatti *et al.*, 1987). The protocol provided by Dr. A. Pfitzner suggested that if possible feeder cells should be avoided as they introduced an unnecessary source of contamination. We did preincubate *L. esculentum* cotyledon explants for 24 hours on MZOZR medium containing acetosyringone, the compound is known to induce the *vir* gene. Co-cultivation with *Agrobacterium* cultures then proceeded for 48 hours on the same MZOZR plates. Immediately prior to co-cultivation, the *Agrobacterium* cultures were grown in *Agrobacterium* induction medium which also contained acetosyringone. However, Fillatti *et al.* (1987) reported that acetosyringone did not appear to be a substitute for tobacco feeder cells. In conclusion, evidence suggests that the use of feeder cells for the preincubation and

co-cultivation of *L. esculentum* explants may have improved the efficiency of the attempted *L. esculentum* transformations.

As a result of the *N. tabacum* SR1 transformation experiments, a number of kanamycin resistant plants were regenerated. The kanamycin resistance gene was contained within the T-DNA region of both the binary vectors used. However, resistance to the drug did not necessarily indicate that the desired insert has been correctly integrated into the plant genome. Only 8 line X plants were regenerated following attempts to transform *N. tabacum* SR1 plants with the replication construct from pBU-XM. PCR analysis suggested that 3 of these (X5, X7, X8) were transgenic (Figure 6.5). Following the attempt to transform *N. tabacum* SR1 plants with the replicase construct from pBTRepTerm, 17 line B plants were regenerated. PCR analysis revealed that 4 (B1, B2, B7 and B8) out of 8 plants chosen for analysis appeared to be transgenic (Figure 6.5). As previously explained in Section 4.3, PCR analysis can be useful for the quick identification of transgenic plants. However, the sensitivity of the technique can lead to either false positives or false negative results. PCR analysis was therefore carried out with the intention of confirming selected plant lines, at a later date. This could be achieved by northern or Southern blot analysis using either RNA or DNA extracted from the plants. The use of different binary vectors made the direct comparisons of transformation efficiencies impossible. Only the replication construct maintained the ability to replicate, a process heavily reliant on host cells. As a result, it may have been more toxic than the replicase construct. This may have decreased the frequency at which transformed cells regenerated to become healthy plants. The *N. tabacum* SR1 explants all failed to develop extensive callus and shoot formation. This was highlighted by comparing the explants with *N. tabacum* Samsun *NN* explants which had been co-cultivated with *Agrobacterium* containing the 35S-GUS construct, pMKC 6 (Section 7.2.4.1). The result could suggest that not even the TMV-based replicase construct was completely benign. It is interesting to note that the regenerated transgenic plants did not appear to demonstrate the symptoms associated with TMV infection, especially as both the TMV encoded replicase proteins and coat protein have been implicated in symptom formation (Sections 1.7.1; 1.7.3; Culver *et al.*, 1991). It is possible that expression of the proteins remained below a threshold level required for symptom formation.

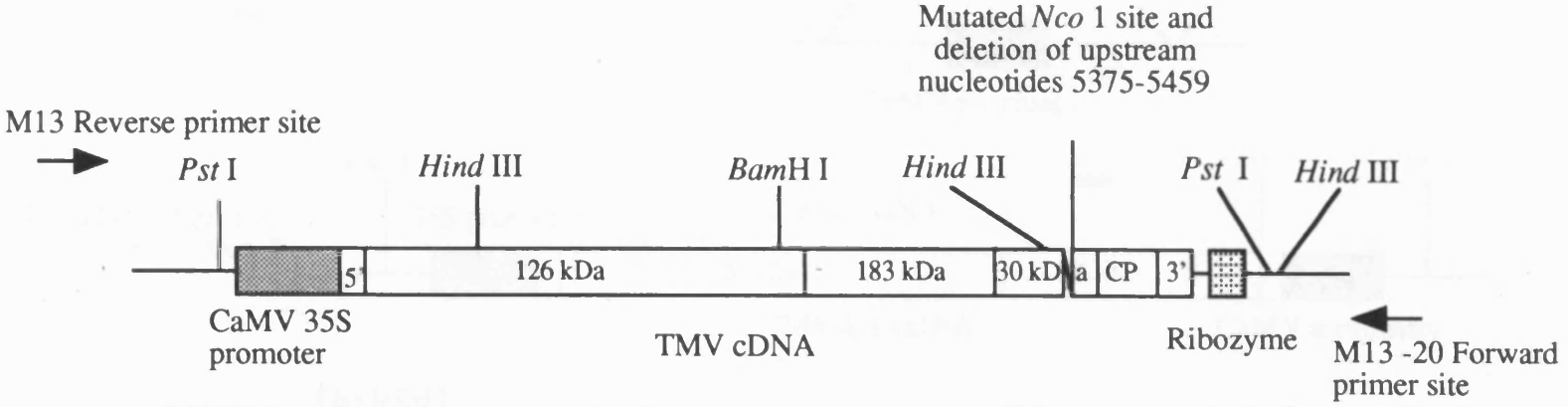
The replicase construct from pBTRepTerm was designed to efficiently express the TMV encoded replicase proteins. However, expression of the proteins would have been dictated by the number of copies integrated into the plant genome of line B plants and the positions at which the integrations took place. The RNA transcribed by the replication construct from pBU-XM should have maintained the ability to replicate. This

may have allowed the TMV proteins, encoded by the construct, to be expressed at high levels despite the variation in copy number and position effects. Instead, expression may have been dictated by sequences within the TMV genome which normally allow the virus to reach high titres in host cells (Section 1.6.8; Dawson and Lehto, 1990). The reason behind the inability to detect the 126 kDa TMV replicase protein in samples taken from transgenic line B and line X plants by western immunoblot analysis is unknown (Figure 6.6). The protein was easily detected in the TMV infected *N. tabacum* SR1 protein sample which was used as a positive control. It is possible that the method was not sensitive enough to detect the replicase protein, especially in line B plants which had been transformed with the replicase construct. TMV coat protein was only clearly detected in protein samples prepared from plants X5 and X8. The level of protein detected was lower than in a sample prepared from a *N. tabacum* SR1 plant which had been manually inoculated with purified TMV particles (Figure 6.7). This could suggest that the replication construct was not expressing TMV encoded proteins in the desired manner. The TMV sequence deleted from pBU-XM is not considered to form part of the LMC-RNA subgenomic promoter (Dawson, *et al.*, 1989 Dawson and Lehto, 1990). However it may still be involved with regulating the expression of coat protein. RNA, complementary to part of the TMV genome, should have been expressed in all the transgenic *N. tabacum* SR1 line X and line B plants. In particular, high levels of RNA were expected in line X plants . However, RNA dot blots failed to detect RNA complementary to TMV nucleotides 1446-5080 in sap extracts prepared from these plants. As only very crude sap extracts were used for the dot blots, it is plausible that the naked RNA was degraded prior to hybridisation. It is anticipated that TMV RNA would have been detected if samples were carefully prepared for northern blot analysis.

Reports of viral resistance in transgenic plants which express TMV RNA or proteins are discussed in Section 1.9.6. The replication construct, from pBU-XM, should have been capable of expressing three proteins which have been reported to confer a resistant phenotype to transgenic plants. Maybe the most well known is the TMV coat protein (Powell Abel *et al.*, 1986; Nelson *et al.*, 1987; Register and Beachy, 1988; Wisniwiski *et al.*, 1990). Expression of the putative 54 kDa protein (Golemboski *et al.*, 1990; Carr and Zaitlin, 1991; Carr *et al.*, 1992) and a dysfunctional 30 kDa protein (Lapidot *et al.*, 1993; Cooper *et al.*, 1995) have also been reported to induce resistance. pBTRepTerm is similar to the two constructs described by Donson *et al.* (1993), which were used to transform *N. tabacum* Xanthi in order to study replicase mediated resistance to tobamoviruses. Surprisingly only one of the constructs led to the regeneration of plants which displayed a resistant phenotype. Analysis of the resistant plants revealed that a transposable element had inserted at

nucleotide 2875 of the TMV-U1 genome. As a result, only a truncated replicase protein was expressed (Donson *et al* 1993). By comparison, it may be expected that if line B plants were resistant to TMV expression of the replicase proteins may not be occurring in the manner intended. The preliminary experiment, designed to investigate possible resistance to TMV in *N. tabacum* SR1 line X and line B plants, indicated that plants X3, X6, B1, B2, B6 and B8 may have been resistant. Leaves from only 7 plants were chosen for analysis and it has already been pointed out that the inoculations may have been unsuccessful. Conclusions cannot yet be drawn from the preliminary results presented in Section 6.2.3.3. However, the results do highlight the importance of conducting further investigations.

Despite differing from the original objectives, a number of interesting results have been presented as a result of the experiments described in this Chapter. In particular transgenic *N. tabacum* SR1 lines have been created. Further analysis of these lines is important so that their ability to express the desired RNAs and proteins can be determined. Such information may be useful for interpreting the results of future successful *L. esculentum* transformation experiments. Furthermore, the analysis of line X plants, transformed with the replication construct from pBU-XM, may provide important information concerning the replicating-marker construct pTMVLUR (Chapter 5). pTMVLUR contained an identical deletion in the TMV-U1 30 kDa protein ORF. Both transgenic plant lines have been considered as controls for the *N. tabacum* Samsun *NN* transformation experiments detailed in Chapter 7. The experiments were aimed at investigating virus-host interactions involved in *N* gene mediated resistance to TMV. The constructs were not designed to investigate resistance, conferred by sequences from the TMV genome, in transgenic plants. However, further analysis to determine the extent of any resistance may contribute to understanding this phenomenon.



pBU-XM, in the binary vector pPZP111

Figure 6.1 Diagram of the Replication Construct pBU-XM. The deletion in the 30 kDa protein ORF has been indicated together with restriction enzyme sites used for cloning and diagnosis.

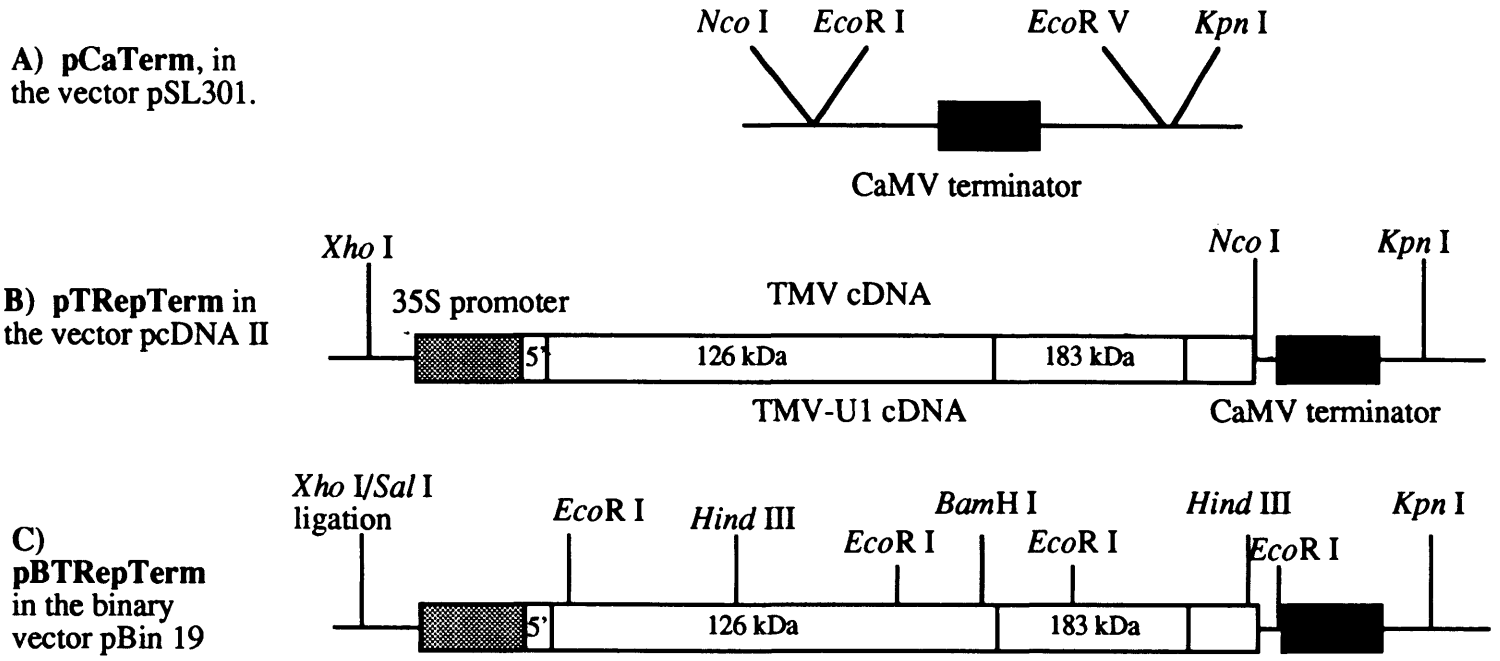
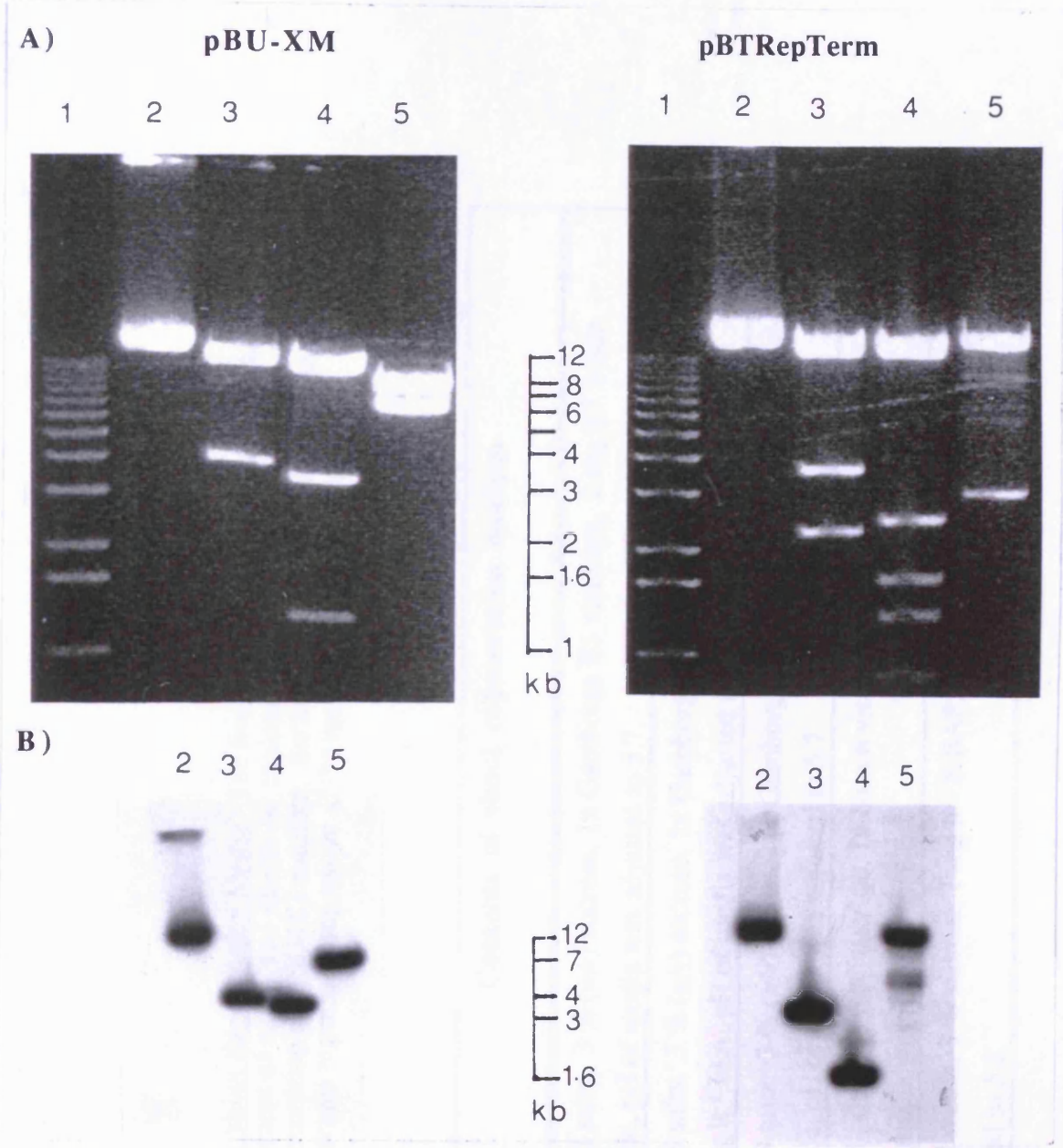


Figure 6.2 Diagram Showing the Construction of pBTRepTerm. The CaMV terminator sequence was cloned into pSL301 (A). It was then used to replace the 3' end of pC-35STMVR (B). Finally the construct was transferred to the binary vector pBin 19. Restriction enzyme sites used for cloning and diagnosis have been indicated.



Lane	Enzyme	Expected fragment size
3	*Bam*H I	12 kb, 4 kb*
4	*Hind* III	11.1 kb, 3.6 kb*, 1.3 kb
5	*Pst* I	8.9 kb, 7.1 kb*

Lane	Enzyme	Expected fragment size
3	*Hind* III	11.1 kb, 3.6 kb*, 2.2 kb
4	*Eco*R I	11 kb, 2.4 kb, 1.6 kb*, 1.2 kb, 0.7 kb
5	*Bam*H I *Kpn* I	14 kb*, 2.9 kb

Figure 6.3 Restriction Enzyme Digests of pBU-XM and pBTRepTerm. DNA fragments were visualised on agarose gels (A). Southern blots were then probed with an ~700 bp *Sst II*, *Bam*H I fragment isolated from pC-35STMVR and exposed to X-ray film for 4 hours (B). Molecular weight markers (lanes 1), Undigested plasmid (lanes 2). The expected DNA fragment sizes (lanes 3-5) are displayed in tables (C). * indicates fragments to which the probe hybridised. Note restriction enzyme digests of pBU-XM were used to orientate the insert within the vector.

Table 6.1 The Contents of Different Shoot Regeneration Media. The five types of media were chosen to investigate optimum conditions for the development of callus and shoots on explants prepared from 8 day old *L. esculentum* GCR 26 (+/+) cotyledons. With the exception of MSD4X2 medium, the ingredients of all media was purchased from Sigma Chemical Company. Further details regarding preparation of the media is given in section 2.12.

Name of shoot regeneration medium	Contents of shoot regeneration medium
A Pfitzner	1x MS salts, 3 % (w/v) sucrose, 1x Gamborgs B5 vitamins, 2 mg lt^{-1} zeatin riboside, pH of media was adjusted to 5.7.
B Pfitzner -a	1x MS salts, 3 % (w/v) sucrose, 1x Gamborgs B5 vitamins, 2 mg lt^{-1} zeatin riboside, 0.5 mg lt^{-1} IAA, pH of media was adjusted to 5.7.
C Pfitzner-b	1x MS salts, 3 % (w/v) sucrose, 1x Gamborgs B5 vitamins, 0.5 mg lt^{-1} zeatin riboside, pH of media was adjusted to 5.7.
D Harrison	1x MS salts, 2 % (w/v) sucrose, 1x Nitsch vitamins, 2 mg lt^{-1} zeatin riboside, pH of media was adjusted to 6.0.
E MSD4X2	1x MS media, 3 % sucrose, 1 mg lt^{-1} 6-BAP, 0.1 mg lt^{-1} NAA, pH of media was adjusted to 5.8.

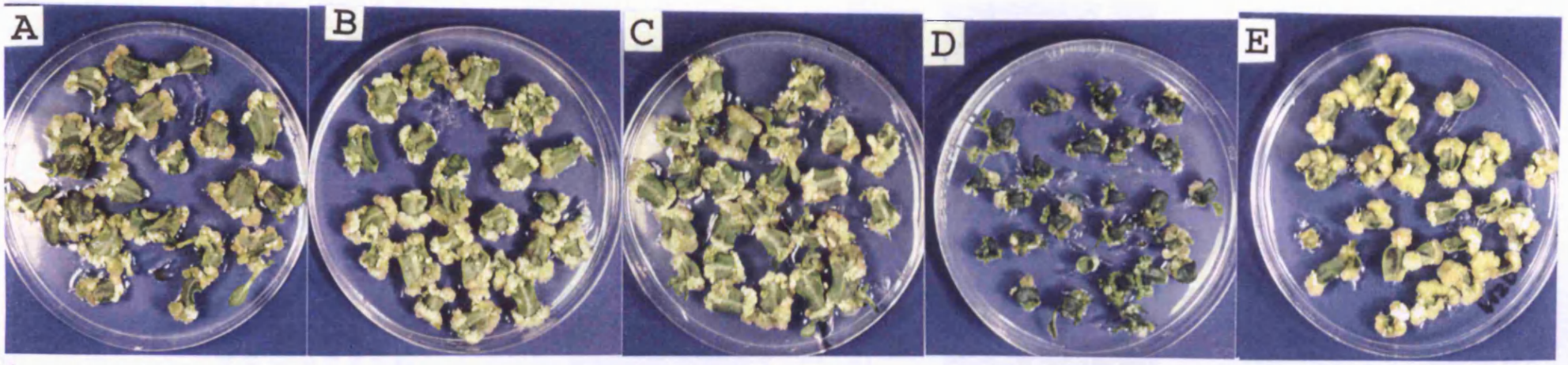


Figure 6.4 Comparison of *L. esculentum* GCR (+/+) Callus and Shoot Formation on Five Different Types of Media. Explants prepared from 8 day old cotyledons were incubated for 28 days, prior to photography, on Pfitzner medium (A); Pfitzner-a medium (B); Pfitzner-b medium (C); Harrison medium (D); MSD4X2 (E). The contents of media is detailed in Table 6.1.

Table 6.2 Comparison of *L. esculentum* GCR 26 (+/+) Shoot Formation on Five Different Types of Media. Explants were prepared from 8 day old cotyledons. The number of shoots was recorded following the incubation of explants for 28 days.

Name of shoot regeneration medium	Number of shoots
A Pfitzner	49
B Pfitzner-a	52
C Pfitzner-b	38
D Harrison	47
E MSD 4x2	11

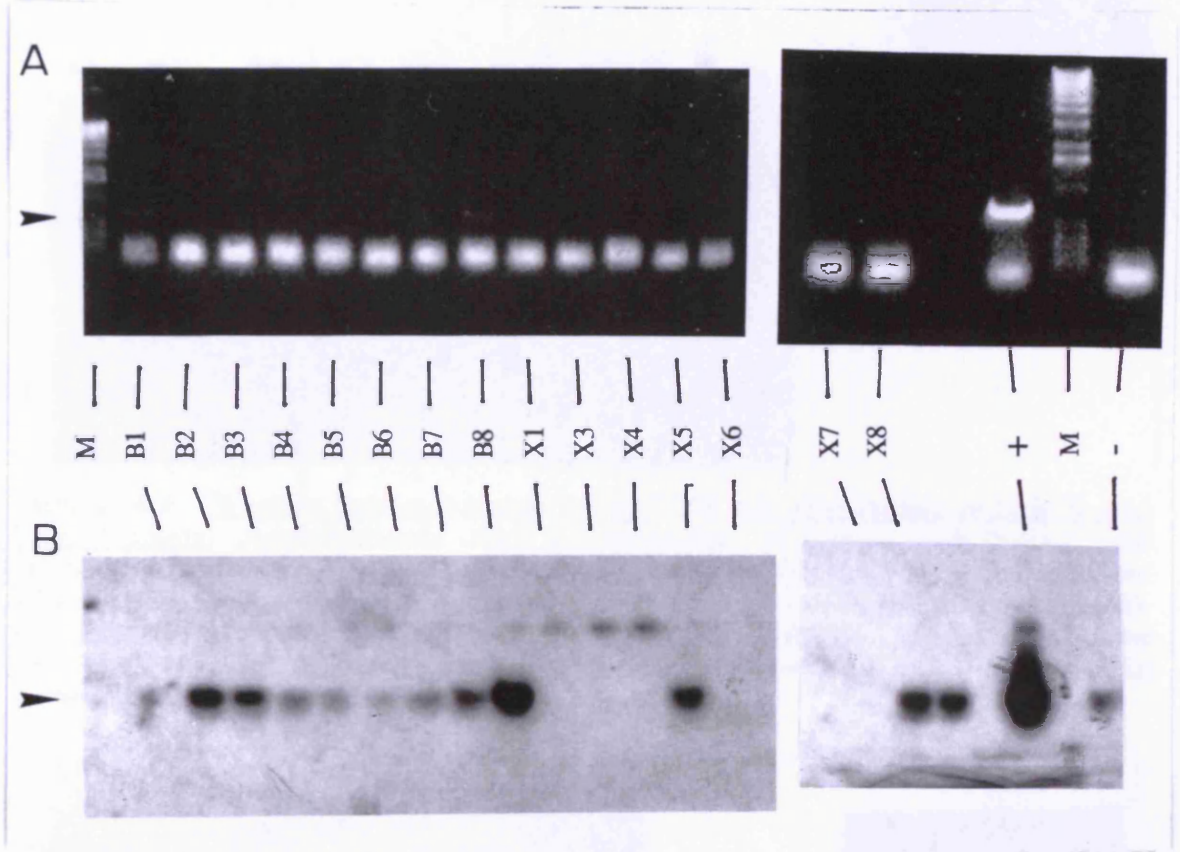


Figure 6.5 PCR Analysis of *N. tabacum* SR1 Line X and Line B Plants. The primers 5'REP and 3'REP were used to amplify a 735 bp fragment from line X plants, transformed with the replication construct from pBU-XM; line R plants, transformed with the replicase construct from pBTRepTerm. Agarose gels showing PCR products (A). Southern blots probed with an ~700 bp *Sst* II, *Bam*H I fragment isolated from pC-35STMVR (B). The blots were exposed to X-ray film for 4 hours. Molecular weight markers (M). PCR using the construct pC-35STMVR (+); genomic DNA from an untransformed *N. tabacum* SR1 plant (-). PCR amplified fragments are indicated by the arrows.

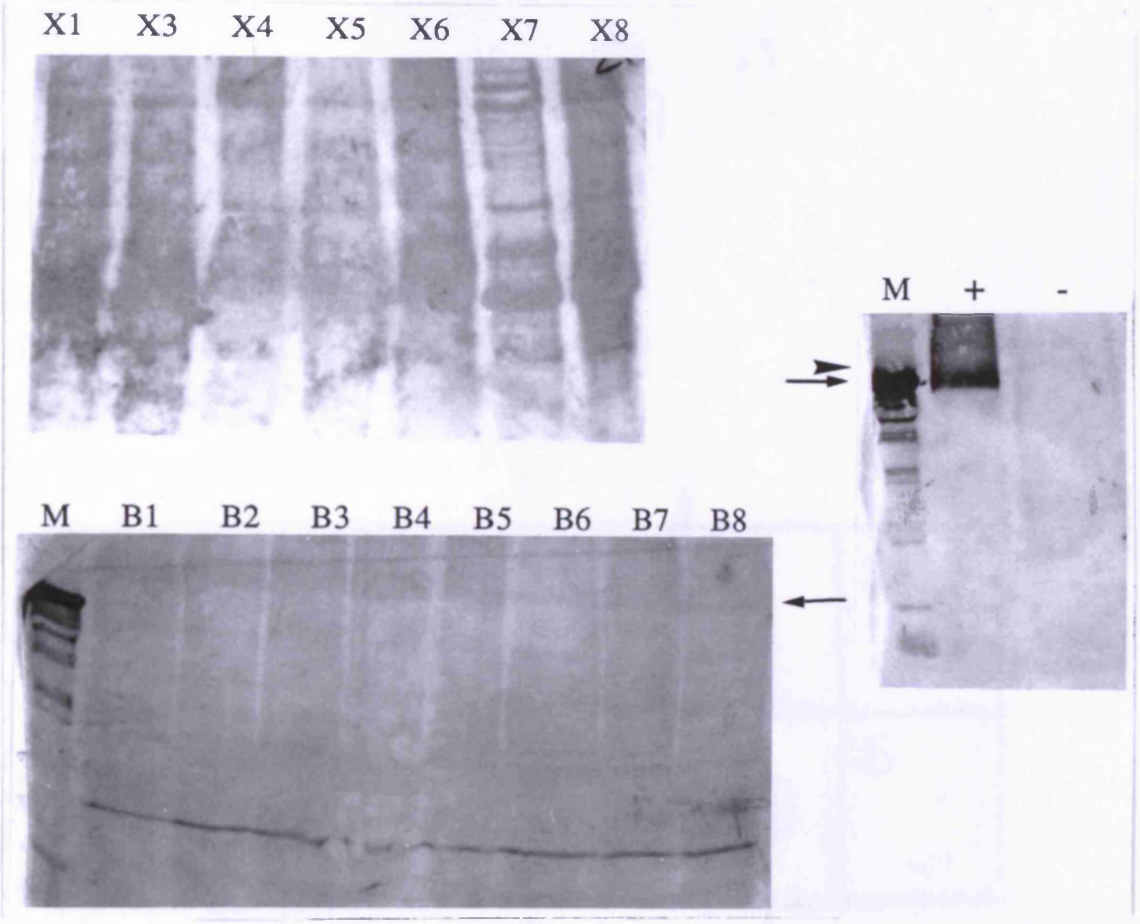


Figure 6.6 Western Immunoblots to Detect TMV 126 kDa Protein in Line X and Line B plants. Approximately equal concentrations of protein were probed with antibody raised to the TMV-U1 126 kDa replicase protein and visualised using an alkaline phosphatase conjugated secondary antibody. Molecular weight markers (M). Protein prepared from a TMV infected *N. tabacum* SR1 plant (+); healthy *N. tabacum* SR1 plant (-). 116 kDa molecular weight marker (⟶), TMV 126 kDa protein (➤).

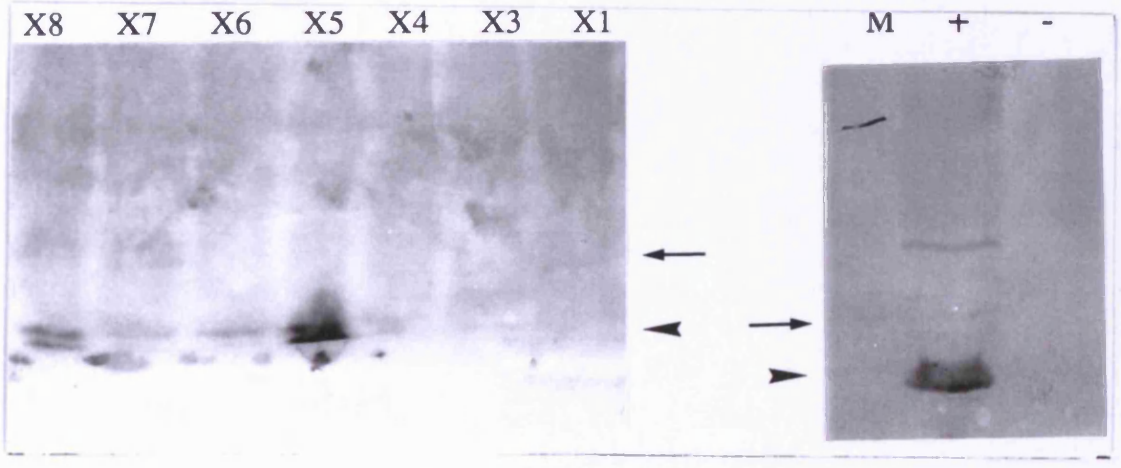


Figure 6.7 Western Immunoblots to Detect TMV Coat Protein in Line X Plants. Approximately equal concentrations of protein, were probed with antibody raised to the TMV-U1 17.5 kDa coat protein and visualised using an alkaline phosphatase conjugated secondary antibody. Molecular weight markers (M). Protein prepared from a TMV infected *N. tabacum* SR1 plant (+); healthy *N. tabacum* SR1 plant (-). 24 kDa molecular weight marker (⟶), TMV 17.5 kDa coat protein (➤).

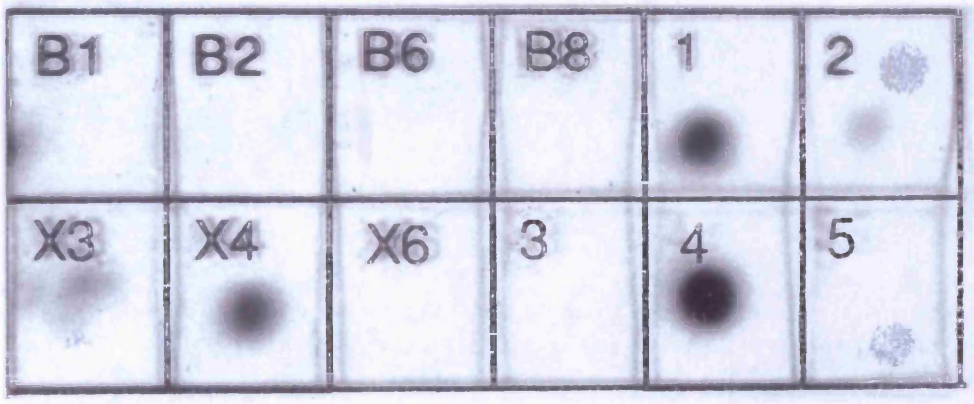


Figure 6.8 Dot Blot to Detect TMV RNA in the TMV Inoculated Leaves of Line X and Line B Plants. Detached leaves were manually inoculated with ~5 µg of purified TMV particles. Extracts were prepared from leaves at 5 days post inoculation. The blot was probed an ~3.6 kb *Hind* III fragment isolated from pC-35STMVR and exposed to X-ray film for 24 hours. Extract prepared from TMV infected, untransformed *N. tabacum* SR1 leaves (1 and 2); a healthy *N. tabacum* SR1 plant (5). dH_2O (3); purified TMV particles (4).

CHAPTER 7

THE EFFECT OF TMV-BASED CONSTRUCTS ON HOST TISSUE EXPRESSING THE *N* GENE

7.1 INTRODUCTION

The *N* gene, originally from *N. glutinosa* (Holmes, 1929), is an effective temperature-sensitive, dominant gene which confers resistance to TMV (Section 1.9.4). At temperatures below 28°C (Otsuki *et al.*, 1972) it is able to interact with the virus to induce a cascade of events culminating in the HR (see Section 1.9.1; reviewed by Goodman and Novacky, 1994). As a result, TMV is effectively confined to the cells surrounding necrotic lesions and is not able to systemically infect plants. Typical necrotic lesions induced by TMV on a *N. tabacum* Samsun *NN* plant, expressing two copies of the *N* gene, are displayed in Figure 7.1. It is important to notice the light brown concentric rings of necrosis. It has been shown that both viral replication (Otsuki *et al.*, 1972) and the expression of viral protein from subgenomic RNAs (Moser *et al.*, 1988) remains uninhibited until the point when lesions first appear. In addition replication occurs normally in protoplasts, irrespective of temperature (Otsuki *et al.*, 1972). Recently, interest in the *N* gene has been stimulated by its successful cloning, sequencing and transformation into *N. tabacum* SR1 (Whitham *et al.*, 1994) and *L. esculentum* plants (Whitham *et al.*, 1996). Sequence analysis has revealed that it appears to be active in the cell cytoplasm (Whitham *et al.*, 1994; Dinesh-Kumar *et al.*, 1995) While it has been presumed that a gene-for-gene interaction (reviewed by Flor, 1971) is responsible for eliciting the HR, the TMV encoded virulence gene has not been precisely identified. As a result, the way in which the virulence gene product may interact with the host encoded resistance gene product is not understood. The study of coat protein deficient mutants provides evidence that the TMV encoded coat protein does not interact with the *N* gene to induce the HR (Takamatsu *et al.*, 1987; Dawson *et al.*, 1988; Holt and Beachy, 1991). This protein appears to be responsible eliciting the *N'* gene mediated HR (see Section 1.9.5). Evidence suggests that the 30 kDa cell-to-cell movement protein is not, on its own, responsible for eliciting the *N* gene. However, it may have a role in either induction or maintenance (Deom *et al.*, 1991). Studies using the resistance breaking strain, TMV-Ob, revealed that the TMV encoded replicase proteins may interact with the *N* gene product, to induce the HR (Padgett and Beachy, 1993). In particular, a single mutation in the 126 kDa/183 kDa protein ORFs appeared to be responsible for the resistance breaking phenotype (Section 1.9.4).

Chapter 3 describes an infectious 35S-cDNA clone of TMV-U1 (in 3 different plasmid vectors). Microprojectile bombardment and plant transformation experiments were successfully used to express the construct in *N. tabacum* SR1 tissue. Encouraged by the success of these experiments, it was decided to investigate the action of the infectious construct in host tissue expressing the *N* gene. At the same time similar experiments were conducted using the previously described replication and replicase

constructs (see Chapter 6). RNA transcribed by the replication construct was designed to be capable of viral replication (Section 1.6.2) and the production of subgenomic RNA (Section 1.6.6). However a deletion within the 30 kDa ORF meant that it should have been incapable of moving from cell-to-cell (Section 1.7.2) or assembly into virus particles (Section 1.6.7). The replicase construct was designed to efficiently express the TMV-U1 126 kDa and 183 kDa replicases proteins. Removal of the 3' untranslated region ensured that RNA would have been incapable of replication (Sections 1.5.1; 1.6.4). Initially the constructs were used for microprojectile bombardments into host tissue expressing the *N* gene. Intrigued by the results, we decided to use the constructs harboured within binary vectors for plant transformation. It was hoped that the results may help elucidate the viral elicitor of *N* gene mediated resistance to TMV.

7.2 RESULTS

7.2.1 Preliminary Microprojectile Bombardments Experiment

Initial microprojectile bombardment experiments were carried out using two different constructs. The first construct, pC-35STMVR, has been described in Chapter 3 (details concerning construction are given in Sections 3.2.1 and 3.2.2) and consisted of a full length cDNA clone of TMV-U1. Transcription *in vivo* was driven by the 35S promoter, starting with the first nucleotide of the TMV genome. A ribozyme sequence was inserted downstream of the TMV genome to limit the number of non-viral nucleotides at the 3' end of viral transcripts. The second construct pU/mcs (described in Section 4..2.2.2) was only able to express the TMV encoded 126 kDa and 183 kDa replicase proteins. This was due to the removal of all nucleotides downstream from the *Nco* I site, situated at nucleotide 5459 of the TMV-U1 genome. Target *N. tabacum* Samsun *NN* leaf tissue (~2.25 cm^2) was prepared from young leaves <8 cm long. Similar tissue from *N. tabacum* SR1 plants had been used successfully for the microprojectile bombardment experiments described in Sections 3.2.5, 4.2.4 and 5.2.4. Approximately 1.4 μg of each construct, pC-35STMVR and pU/mcs, was independently bombarded into six separate leaf samples. At the same time six control leaf samples were bombarded with tungsten particles, which had not been coated in DNA. The control tissue was used to identify symptoms which could be attributed to the microprojectile bombardment procedure. Following bombardment with pC-35STMVR few lesions appeared, instead mild to severe yellowing symptoms developed over 8 days post bombardment (Figure 7.2). By comparison tissue bombarded with either pU/mcs or tungsten particles in the absence of DNA remained symptomless.

7.2.2 Choice of Constructs and Plants for Further Investigations

It was decided that further microprojectile bombardment experiments were required to determine the ability of pC-35STMVR to elicit the *N* gene-mediated HR. At the same time microprojectile bombardment experiments were designed to investigate the ability TMV-based replication and replicase constructs to elicit the *N* gene. The replication construct pCU-XM has previously been described in Section 5.2.1.2 and 6.2.1. It was similar to pC-35STMVR, however, nucleotides 5375-5459 had been deleted from the TMV-U1 cDNA. These were within the 30 kDa cell-to-cell movement ORF and included an essential part of the OAS sequence. The replicase construct pTRepTerm has been described in Section 6.2.1 and superseded the construct pU/mcs, used in the preliminary microprojectile bombardment experiment. It was identical to pC-35STMVR upstream of the *Nco* I site located at nucleotide 5459 of the TMV-U1 genome. The 3' end of the TMV cDNA, and the ribozyme sequence, had been replaced by a transcription termination and polyadenylation signal from CaMV. A diagrammatic representation of the infectious, replication and replicase constructs used for microprojectile bombardment experiments is presented in Figure 7.3. As the constructs have all been described previously, details of cloning procedures and restriction enzyme sites have been omitted. Plant transformation required that each construct was harboured within a binary vector suitable for *Agrobacterium*-mediated gene transfer. These binary constructs have also been previously described in Sections 3.2.2 and 6.2.1. The binary infectious construct pB-35STMV and binary replicase construct pBTRepTerm were cloned into the vector pBin 19. The binary replication construct pBU-XM was cloned into the vector pPZP111. Essentially the constructs were the same as those described above (and presented in Figure 7.3). However, pB-35STMV no longer contained a ribozyme sequence downstream from the 3' end of the TMV cDNA.

Both microprojectile bombardment and plant transformation experiments were carried out using *N. tabacum* Samsun *NN*. We also had access to transgenic *N. tabacum* Samsun *NN* PR-1a-GUS (line 8) plants (Bi *et al.*, 1995), these had been transformed using a construct supplied by Uknes *et al.* (1993). As a result, the plants were able to express the GUS gene under the control of the pathogen related protein 1a (PR-1a) promoter. Expression of the acidic PR-1 proteins (a, b and c) is induced as part of the HR (Section 1.9.1). The proteins have been shown to be concentrated in the area immediately surrounding lesions (Heitz *et al.*, 1994). Following microprojectile bombardment of constructs into transgenic *N. tabacum* Samsun *NN* PR-1a-GUS leaf tissue, it was possible to carry out either fluorimetric or histochemical GUS assays to identify PR-1a promoter activity. This reflected the *N* gene-mediated

HR induced by the constructs. Production of the PR-1a protein can also be induced by stress (Ohshima *et al.*, 1990). Microprojectile bombardment experiments were therefore designed to include controls which would help indicate stress induced PR-1a promoter activity.

7.2.3 Microprojectile Bombardment Experiments

7.2.3.1 Bombarding *N. tabacum* Samsun *NN* Tissue

It was thought that the absence of lesions in the preliminary microprojectile bombardment experiments may have been due to the small size (~2.25 cm^2) of target tissue. As a result further bombardments were carried out using larger tissue samples, ~9 cm^2. Target tissue samples, prepared from *N. tabacum* Samsun *NN* plants, were bombarded with either the infectious construct pC-35STMVR, replication construct pCU-XM, replicase construct pTRepTerm or control plasmid pBS SK^-. Symptoms were left to develop for 4 days post bombardment before being photographed (see Figure 7.4). Lesions and in some cases yellowing started to appear at 2-3 days post bombardment with the infectious construct pC-35STMVR and were clearly visible by 4 days post bombardment. This proved to be the optimum time for viewing as deterioration of the detached leaf tissue began to obscure the defined lesions by 5 days post bombardment. Tissue bombarded with either pCU-XM, pTRepTerm or pBS SK^- remained essentially symptomless. Small areas of necrosis or yellowing did appear on tissue bombarded with pBS SK^-. These corresponded to the most heavily bombarded areas (judged by tissue damage and the grey appearance of tungsten particles) and suggested that damage caused by the procedure induced only mild visual symptoms. The bombardment of *N. tabacum* Samsun *NN* tissue with the infectious constructs pC-35STMVR, replication construct pTRepTerm and control plasmid pBS SK^- was repeated. Tissue was incubated for 6 days post bombardment and then used to prepare protein samples for western immunoblot analysis. Approximately equal concentrations of protein were probed with antibody raised to the PR-1a protein (Section 2.13.2.1), the antibody was known to cross react-with PR-1 proteins b and c (Antoniw *et al.*, 1985). Visualisation was carried out using an alkaline phosphatase conjugated secondary antibody (Figure 7.5). The resolution of the 12 % polyacrylamide mini gel was not sufficient to separate the PR-1 proteins a, b and c, which are all ~15 kDa. As a result, only one band indicating the presence of all three PR-1 proteins was expected. Upon development only faint bands were present in protein samples prepared from tissue bombarded with either pTRepTerm or pBS SK^-. However, a band, clearly distinguishable from background, was present in the sample bombarded with pC-35STMVR.

7.2.3.2 Bombarding Transgenic *N. tabacum* Samsun *NN* PR-1a-GUS Tissue

It was decided to bombard the constructs into transgenic *N. tabacum* Samsun *NN* PR-1a-GUS tissue, ~9 cm^2, prepared from leaves of mixed age. Quantitative fluorimetric analysis was then used to identify levels of PR-1a promoter activity. Each bombardment consisted of ~1.15 μg of the construct being investigated and ~0.25 μg of the control construct pRTS2-LUC (Turner *et al.*, 1994). This allowed the success of each type of bombardment to be estimated. PR-1a promoter activity in tissue bombarded with either pCU-XM or pTRepTerm was expected to be only slightly higher than in tissue bombarded with pBS SK^-. It was therefore desirable to perform fluorimetric analysis only on the tissue hit by tungsten particles, and not the surrounding unbombarded tissue. Both luciferase and GUS analysis was carried out using samples ~2.25 cm^2, prepared from the ~9 cm^2 target samples. Attempts were made to select tissue within the distribution zone of the tungsten particles. This was easily identified by the appearance of lesions following the bombardment of pC-35STMVR. Identification of the distribution zone was harder following the microprojectile bombardment of either pCU-XM, pTRepTerm or pBS SK^-. However, it was sometimes possible to identify areas of tissue damage, or a slight grey coloration indicating the presence of tungsten particles. Due to the unstable nature of the luciferase enzyme (Section 3.2.5), luciferase assays were carried out at 24 hours post bombardment, using a proportion of the bombarded tissue. This tissue was no longer available for GUS analysis at 4 days post bombardment. As a result, luciferase activities were used only to estimate the viability of each type of bombardment, and were not included in calculations of relative activities.

The infectious construct pC-35STMVR, replication construct pCU-XM, replicase construct pTRepTerm and control plasmid pBS SK^- were independently bombarded into 4 tissue samples. One sample, from each type of bombardment, was selected and analysed for luciferase activity at 24 hours post bombardment. The light units emitted upon the addition of luciferin and ATP were pC-35STMVR 128 450 LU; pCU-XM 110 290 LU; pTRepTerm 519 820 LU and pBS SK^- 375 630 LU. These luciferase activities, together with the development of lesions on tissue bombarded with pC-35STMVR, indicated that the bombardments had been successful. The symptoms observed at 4 days post bombardment with either pC-35STMVR, pCU-XM, pTRepTerm or pBS SK^- were comparable to those photographed in Figure 7.4. The remaining three samples from each type of bombardment were assayed to determine their GUS activity (calculated according to the method described in Section 2.13.4.1). The activities, reflecting levels of PR-1a promoter activity, have been presented in Table 7.1 and Figure 7.7. As expected the mean activity was highest in tissue bombarded

with the infectious construct pC-35STMVR, this was calculated to be 127.6 pMol 4MU min^{-1} mg $protein^{-1}$. Mean activities calculated in tissue bombarded with the replication construct pCU-XM and the replicase construct pTRepTerm were much lower at 7.87 pMol 4MU min^{-1} mg $protein^{-1}$ and 19.02 pMol 4MU min^{-1} mg $protein^{-1}$ respectively. These activities were also lower than the activity of 71.62 pMol 4MU min^{-1} mg $protein^{-1}$ calculated for tissue bombarded with control plasmid pBS SK^-. This high activity in tissue bombarded with pBS SK^- appears to be due to one bombarded sample, it is unclear why the activity was so high in this sample.

7.2.3.3 Further Optimisation of the Bombardment Procedure

The preparation of target tissue samples ~9 cm^2, as opposed to 2.25 cm^2, required the use of larger and therefore older leaves. Previous successful microprojectile bombardment experiments, described in Sections 3.2.5, 4.2.4 and 5.2.4, all made use of young leaves <8 cm long. By studying symptoms induced on older tissue following bombardment with pC-35STMVR, it was apparent that the response may have been influenced by the age of target tissue. This was investigated further using the infectious construct pC-35STMVR and transgenic *N. tabacum* Samsun *NN* PR-1a-GUS leaf tissue. Detached leaf samples ~9 cm^2 were prepared from young leaves <8 cm long, developing leaves 10-12 cm long and mature leaves >15 cm long. Care was taken to choose healthy dark green leaves, in the case of mature leaves these were the most recent to mature. pC-35STMVR was bombarded into 4 samples prepared from each type of leaf. Symptoms were allowed to develop over 4 days post bombardment. Two leaf samples from each type of leaf were then photographed and histochemically stained, the results are presented in Figure 7.8. The remaining two samples from each types of leaf appeared similar to those photographed and were used for fluorimetric analysis. Young leaf tissue developed fewer and smaller lesions following microprojectile bombardment with pC-35STMVR. This tissue also appeared to be more prone to yellowing, although this was most apparent on the bombarded young leaf tissue photographed in Figure 7.2. This observation is supported by Figure 7.6, which shows a detached *N. tabacum* Samsun *NN* leaf, ~6 cm long, at 7 days post manual inoculation with ~5 µg of purified TMV particles. Symptoms on the leaf, in particular a yellow phenotype, bear little resemblance to the characteristic TMV induced lesions pictured in Figure 7.1. One young unstained leaf sample, pictured in Figure 7.8E, displayed two areas of necrosis. These were attributed to tissue damage as they did not take the appearance of defined, TMV induced, lesions. Furthermore following histochemical staining, cells surrounding the necrosis failed to develop a blue pigment. This would have indicated that PR-1a promoter activity, induced as part of the HR, was driving expression of the GUS gene.

Heavy staining developed on the second young leaf sample (Figure 7.8F) as a result of microprojectile bombardment with pC-35STMVR. The response displayed in the developing leaf samples as a result of microprojectile bombardment with pC-35STMVR was slightly improved (Figure 7.8, C and D). However, the lesions were still small and histochemical staining failed to indicate high levels of PR-1a promoter activity in one of the samples. Lesions were clearly visible on both mature leaf samples at 4 days post bombardment of pC-35STMVR (Figure 7.8, A and B). These lesions appeared to be much darker than the characteristic TMV induced lesions pictured in Figure 7.1. The area surrounding the lesions, on the mature leaf samples, developed a dark blue pigment upon histochemical staining. The conclusions drawn from visual observations were confirmed following fluorimetric analysis of two samples from each leaf type. The results are presented in Table 7.2 and Figure 7.9. The expression of GUS, indicating PR-1a promoter activity, was detected in all leaf samples as a result of microprojectile bombardment with pC-35STMVR. The highest activity was detected in samples taken for mature leaves, the average activity being 472.30 pMol 4MU min^{-1} mg $protein^{-1}$. This was compared with average activities of 148.01 pMol 4MU min^{-1} mg $protein^{-1}$ and 120.58 pMol 4MU min^{-1} mg $protein^{-1}$ for samples prepared from developing and young leaves, respectively. In conclusion, it appeared that mature target leaf tissue was the most suited to further microprojectile bombardment experiments, designed to investigate induction of the *N* gene-mediated HR by TMV-based cDNA constructs.

One final experiment was conducted prior to proceeding with microprojectile bombardments using the infectious, replication and replicase constructs. The construct pC-35STMVR was bombarded into large (~9 cm^2) and small (2.25 cm^2) target tissue prepared from mature *N. tabacum* Samsun *NN* PR-1a-GUS leaves. Following histochemical staining of tissue at 4 days post bombardment lesions appeared on both sizes of tissue (Figure 7.10). Obviously, the frequency of the plasmid coated tungsten particles being successfully delivered into target tissue was increased when larger target tissue samples were used.

7.2.3.4 Final Analysis of the Infectious, Replication and Replicase Constructs

Taking into consideration the results presented in Section 7.2.3.3, final qualitative analysis of the constructs was carried out using tissue ~9 cm^2 prepared from mature transgenic *N. tabacum* Samsun *NN* PR-1a-GUS leaves. Tissue was bombarded with either the infectious construct pC-35STMVR, replication construct pCU-XM, replicase construct pTRepTerm or control plasmid pBS SK^-. Only tissue bombarded with pC-35STMVR developed visible lesions which were similar to those

pictured on the mature leave tissue in Figure 7.8. It was thought that histochemical staining may indicate PR-1a promoter activity in the tissue surrounding single cells eliciting the HR. The staining procedure was carried out at 4 days post bombardment (Figure 7.12). The tissue surrounding lesions on samples bombarded with pC-35STMVR developed a dark blue pigment. This indicated that infectious TMV particles transcribed by pC-35STMVR were eliciting the *N* gene-mediated HR. Although tissue bombarded with either pCU-XM or pTRepTerm developed a blue pigment, it was indistinguishable from the pigment in tissue bombarded with the control plasmid pBS SK$^-$. The pigment tended to be in circles, ~1.5 cm diameter, suggesting that this was the distribution of tungsten particles delivered using the optimised condition described in Section 2.11.2.2. The results indicated that stress, introduced by the bombardment process, induced some PR-1a promoter activity. This may explain the faint bands observed following the western immunoblot analysis of *N. tabacum* Samsun *NN* tissue bombarded with either pTRepTerm and pBS SK$^-$ (Figure 7.5). Levels of stress induced PR-1a promoter activity varied in individual tissue samples following bombardment with the same construct. However it was particularly high in leaf veins even when they were outside the ~1.5 cm diameter putative tungsten distribution zone.

Two further microprojectile bombardment experiments were carried out using transgenic *N. tabacum* Samsun *NN* PR-1a-GUS leaf tissue. It was intended to obtain quantitative data showing levels of GUS and as a result PR-1a promoter activity. Lesions failed to develop on tissue at 4 days post bombardment with pC-35STMVR. Furthermore the results of luciferase and GUS assays were low, indicating that the bombardments had not been successful. Meaningful conclusions could not be drawn from the poor results and as a result they have not been presented.

7.2.3.5 Microprojectile Bombardment of pTMVB-LUR

The replication-marker construct pTMVB-LUR has been described in Chapter 5. It consisted of a 35S-cDNA clone of TMV with almost the entire coat protein gene replaced by the luciferase marker gene (Figure 5.7). As a result, transcribed RNA should have been capable of replication (Section 1.6.2) and cell-to-cell movement (Section 1.7.2). It should not have been coated into virus particles or have been capable of efficient systemic movement (Section 1.7.3). TMV mutants which lack the coat protein gene, yet still replicate and move from cell-to-cell, maintain the ability to induce lesions on tissue expressing the *N* gene (Section 1.9.4; Takamatsu *et al.*, 1987; Dawson *et al.*, 1988; Holt and Beachy, 1991). In order to complement the microprojectile bombardment experiments described in Section 5.2.4, pTMVB-LUR

was bombarded into three transgenic *N. tabacum* Samsun *NN* PR-1a-GUS tissue samples ~9 cm^2, prepared from mature leaves. No lesions were observed at 4 days post bombardment. Histochemical staining (Figure 7.11) revealed that PR-1a promoter activity appeared to be similar to the activity observed in tissue bombarded with pBS SK^- (Figure 7.12). No further bombardments were carried out to investigate the activity of pTMVB-LUR in tissue expressing the *N* gene.

7.2.4 Plant Transformation

7.2.4.1 Regeneration of Transgenic Plants

It was decided to use plant transformation to continue investigating induction of the HR by the TMV-based infectious, replication and replicase constructs. The binary infectious construct pB-35STMV, replication construct pBU-XM and replicase construct pBTRepTerm were used to transform *N. tabacum* Samsun *NN*. Each binary construct has been previously described in Sections 3.2.2 and 6.2.1 respectively. They were similar to the constructs used for microprojectile bombardment experiments (Section 7.2.2; Figure 7.3). A control binary 35S-GUS construct, pMKC 6, was also transformed into *N. tabacum* Samsun *NN* in order to confirm the success of the transformation procedure. The construct was built in the binary vector pBin 19 by Man-Kim Cheung (Department of Botany, University of Leicester). It consisted of the CaMV 35S promoter, tobacco etch virus (TEV) translational leader sequence, CaMV 35S polyadenylation signal from pRTL2 (Restrepo *et al.*, 1990) and a GUS-intron construct generated by Vancanneyt *et al.* (1990). The intron was inserted into the GUS gene to prevent expression of the gene by *Agrobacterium*. All binary constructs were conjugated into *A. tumefaciens* strain LBA4404 and co-cultivated independently with ~120 *N. tabacum* Samsun *NN* explants.

Clear differences between the appearance of explants were observed by 21 days post co-cultivation with the *Agrobacterium* cultures. These were recorded at 28 days post co-cultivation and the results are presented in Figures 7.13 and 7.14 and Tables 7.3 and 7.4. The plates were kept for a further 8 weeks, during this time calli and shoots continued to develope. However, despite slight deterioration, the appearance of the explants did not significantly change. Following co-cultivation with *Agrobacterium* harbouring the infectious construct, pB-35STMV, 49 % of the *N. tabacum* Samsun *NN* leaf disc explants developed necrotic symptoms. In the majority of cases this was a light brown necrosis. The concentric rings of necrosis, normally visible in lesions induced by TMV on plants expressing the *N* gene (Figure 7.1), were not apparent. In some cases the entire leaf disc was necrotic, other leaf discs contained both necrotic and

healthy green areas. Black/dark brown necrosis developed on 6 % of leaf discs. RNA dot blots were carried out using tissue from black, light brown and green leaf discs at 8 weeks post co-cultivation. They were probed with an ~3.6 kb *Hind* III fragment isolated from pC-35STMVR (nucleotides 1446-5080 of the TMV-U1 genome). TMV RNA was detected in the black and light brown leaf discs, but not in the green leaf discs tissue (Figure 7.15). Three shoots developed on leaf discs co-cultivated with *Agrobacterium* harbouring the infectious construct pB-35STMV. Two shoots grew from light brown leaf discs but developed light brown necrosis and died within a week. The third shoot grew from a green leaf disc. It appeared to be an untransformed escape and as a result failed to root in the presence of kanamycin.

Leaf disc explants co-cultivated with *Agrobacterium* harbouring either the replication construct pBU-XM or the replicase construct pBTRepTerm remained essentially symptomless (see Figures 7.13 and 7.14). Small amounts of necrosis were apparent by 21 days post co-cultivation. It was thought to be due to wounding caused by the transformation procedure. The necrosis was similar in quantity and appearance to the necrosis observed on explants co-cultivated with *Agrobacterium* harbouring the control plasmid pMKC 6. Two shoots were apparent by 28 days, following the co-cultivation of *N. tabacum* Samsun *NN* explants with *Agrobacterium* harbouring the replication construct pBU-XM. Three more shoots developed at a later date. Only three shoots in total developed roots in the presence of kanamycin. The regenerated plants were transferred to soil and genomic DNA was extracted for PCR analysis. The primers 5'REP and 3'REP (described in Section 3.2.6) were designed to amplify a 735 bp fragment from the coding region of the TMV replicase proteins (nucleotides 2485-3219 of the TMV-U1 genome). The result of PCR analysis using these primers suggested that the replication construct from pBU-XM had been stably integrated into the genomes of all three regenerated plants (see Figure 7.17). The transgenic plants were numbered and given the prefix "M". Soon after transfer to soil the regenerated plant M2 developed a light brown systemic necrosis starting at the base of the plant. The plant was photographed shortly before it died along with the regenerated plant M3 (Figure 7.16). The regenerated T_0 plants, M1 and M3, developed without obvious necrotic, chlorotic or mosaic symptoms. However, compared to the regenerated plants transformed using either pBTRepTerm or pMKC 6 their growth was extremely slow. The plants remained stunted, <15 cm tall, having been in soil for over 8 weeks, by this time the other successfully transformed lines were ~60 cm tall and flowering. Following the co-cultivation of *N. tabacum* Samsun *NN* explants with *Agrobacterium* harbouring the replicase construct pBTRepTerm, 18 shoots developed within 28 days. Seven of these shoots developed kanamycin resistant roots and were transferred to soil. The plants were numbered and given the prefix "R". PCR analysis, using primers

5'REP and 3'REP, was used to determine whether the replicase construct from pBTRepTerm had been stably integrated into the plant genomes. Following agarose gel electrophoresis the amplified DNA bands were faint and did not photograph well (Figure 7.17). It was possible to clearly identify bands in genomic DNA samples prepared from plants R6 and R7. Less obvious bands were present in genomic DNA samples prepared from plants R3 and R4. The agarose gel was used for Southern blot analysis and the filter was probed with an ~700 bp *SSt* II, *Bam*H I fragment isolated from pC-35STMVR((nucleotides 2653-3332 of the TMV-U1 genome). The results suggested that all seven regenerated line R plants contained the stably integrated replicase construct (Figure 7.17). It should be noted that PCR analysis can often give rise to false positive or negative results. The described PCR analysis of the T_0 line M and line R plants was intended to be preliminary. Further analysis was to be carried out using T_1 generation plants in order to confirm the results. Following the co-cultivation of *N. tabacum* Samsun *NN* explants with *Agrobacterium* harbouring the control construct pMKC 6 over 115 shoots developed. Only a small selection of these were rooted on kanamycin and grown for analysis. The plants were numbered and given the prefix "G", fluorimetric analysis was used to confirm their transgenic nature. The result suggest that out of the 13 plants analysed, at least 9 expressed higher than background levels of GUS (Table 7.7). The activities ranged from 10-24 330 pMol 4MU min^{-1} mg protein $^{-1}$ and suggested that all 9 plants were transgenic. The variation in activity may reflect both the copy number of the transgene and positions at which transgenes were integrated into the plant genome. Although expression in tissue sampled from plants G5 and G9 and G11 was higher than in tissue samples from the untransformed control *N. tabacum* Samsun *NN* plant, expression was still low. As a result, an element of doubt remained concerning the transgenic nature of these plants.

7.2.4.2 Western Immunoblot Analysis

Western immunoblot analysis was used to determine whether TMV encoded proteins could be detected in the regenerated T_0 line M and line R plants. Of the regenerated line M plants, only M1 was large enough to allow the removal of leaf tissue at the time of analysis. All seven line R plants (R1-R7) were analysed. The western immunoblot experiments were carried out at the same time as those described in Section 6.2.3.2. Controls showing the presence of the TMV encoded 126 kDa replicase protein and the coat protein in TMV inoculated *N. tabacum* SR1 plants are shown in Figures 6.6 and 6.7. The Figures also demonstrate that the TMV encoded proteins were absent in uninoculated tissue. All protein samples prepared from line M and line R plants were probed with antibody raised to the TMV-U1 126 kDa replicase protein. Protein was visualised using an alkaline phosphatase conjugated secondary antibody.

The results are presented in Figures 7.18 and 7.19 and are similar to the results presented in Figure 6.6. Following development for 30 minutes either single or double bands representing proteins >116 kDa were present in all samples. However the proteins were also judged to be larger than the 126 kDa replicase protein. Furthermore, following the analysis of uninfected tissue, a similar faint doublet was observed after development for only 10 minutes (Figure 6.6). RNA transcribed by the replication construct pBU-XM should have been capable of replication and the production of coat protein. Protein prepared from the regenerated plant M1 was therefore probed with antibody raised to TMV-U1 coat protein. Coat protein was not detected following visualisation using an alkaline phosphatase conjugated secondary antibody (see Figure 7.18). As induction of the HR is associated with the production of PR proteins a final western immunoblot was probed with antibody raised to the PR-1a protein (protein prepared from plant R7 was not included in this experiment). Again visualisation was carried out using an alkaline phosphatase secondary antibody. Following development for 30 minutes bands did appear (Figure 7.20). However, bands of a similar size were present in the control sample prepared from an untransformed *N. tabacum* SR1 plant. There were no obvious bands at ~15 kDa to indicate production of the PR-1 proteins (a positive PR-1a protein control was not included).

7.2.4.3 Infection of Transgenic Plants with TMV

It is possible that during the transformation procedure we had selected plants with an impaired HR. Furthermore, there are many reports that transgenic plants expressing part of the TMV genome exhibit resistance to TMV (Section 1.9.6). As a result, we were interested in determining whether the HR was still elicited in the transgenic line M and line R plants and/or whether the plants expressed an additional form of transgene-mediated resistance. In order to allow analysis of the T_1 generation, it was essential that the T_0 plants produced seed. It was therefore decided to inoculate a detached leaf, from some of the transgenic plant lines, with purified TMV-U1 particles. Prior to inoculation, tissue samples from plant lines M1, R1-R7 and G1-G8 were used to prepare RNA dot blots. These were probed with an ~3.6 kb *Hind* III fragment isolated from pC-35STMVR . The procedure failed to detect TMV RNA in any of the regenerated lines (results not presented). The following plants were chosen for the inoculation experiment M1, R1, R4, R6, R7, G1, G2, G4 and G7. A detached leaf from each plant was manually inoculated with ~0.5 μg of purified TMV-U1 particles. Symptoms started to develop at two days post inoculation and were photographed at 5 days post inoculation. The results are presented in Figures 7.22-7.25. Dark brown necrotic lesions developed on the line G leaves which had been transformed with the

35S-GUS construct from pMKC 6 (Figures 7.22; 7.24). The lesions were similar in size and appearance to those pictured in Figure 7.1. However they were much darker, making the concentric rings of necrosis difficult to distinguish. It was thought that this may be due to the fact that the leaves from line G plants were detached prior to inoculation while the leaf displayed in Figure 7.1 remained attached to a plant until photographed. The lesions which developed on the line R leaves, which had been transformed with the replicase construct from pBTRepTerm, were light grey (Figures 7.23; 7.24). They appeared to be slightly smaller than the lesions which developed on line G leaves and concentric rings of necrosis remained hard to distinguish. On the manually inoculated leaf detached from plant R6, distinct yellow symptoms developed in areas not associated with lesion formation. The number of lesions on detached line G and line R leaves varied. It was likely that this was due to minor variations in the inoculation procedure. The most interesting symptoms developed on the detached inoculated leaf from plant M1 - this plant had been transformed with the replication construct from pBU-XM (Figure 7.25). Grey necrosis developed and in most cases did not take the form of the defined lesions which are normally induced by TMV on host tissue expressing the *N* gene. The necrosis was surrounded by severe yellowing. The symptoms developed throughout the entire leaf, while symptoms on line G and line R leaves appeared to be more confined. However, it should be noted that the line M1 leaf was one of the smaller leaves used in this inoculation experiment. Prior to discarding the inoculated leaves, tissue samples were taken separately from both necrotic and healthy areas and used for RNA dot blot analysis. The dot blots were probed with an ~3.6 kb *Hind* III fragment isolated from pC-35STMVR. Unfortunately the samples taken from the two separate areas became mixed and were therefore pooled. However, the results suggest that TMV RNA accumulated at lower levels in the pooled samples prepared from line G leaves (Figure 7.21). Higher levels of TMV RNA were detected in samples prepared from line R and line M leaf tissue.

7.3 DISCUSSION

The *N* gene confers resistance to TMV by inducing the plants' HR (Sections 1.9.1; 1.9.4). As a result, virus is confined to areas immediately surrounding the necrotic lesions which form at sites of infection. The aim of the experiments described within Section 7.2 was to determine the ability of TMV-based cDNA constructs to elicit the *N* gene. Using *N. tabacum* Samsun *NN* and transgenic *N. tabacum* Samsun *NN* PR-1a-GUS plants, it was decided to investigate three different constructs. Each construct was harboured in two separate plasmid vectors. The smaller plasmid vector was used for microprojectile bombardment experiments,

while the larger binary vectors were suitable for *Agrobacterium*-mediated plant transformation (see Figure 7.3). All three constructs should have been capable of expressing the TMV encoded 126 kDa and 183 kDa replicase proteins. A previous report has implicated these proteins as elicitors of the *N* gene-mediated HR (Padgett and Beachy, 1993). A natural progression of the experiments described in Chapter 3 was to determine the ability of the infectious construct, pC-35STMVR, to induce the HR in host tissue expressing the *N* gene. As explained in Section 7.1 the replication construct pCU-XM was similar to the infectious construct except for a deletion within the 30 kDa protein ORF. As a result transcribed RNA should have been capable of replication and the production of subgenomic RNAs but incapable of moving from initially infected cells or assembling into virus-like particles. The replicase construct pTRepTerm should have expressed the TMV encoded replicase proteins. However, transcribed RNA was incapable of replication or the production of subgenomic RNAs.

Some response was observed as a result of the preliminary microprojectile bombardment experiments (Section 7.2.1; Figure 7.2). However, lesions did not develop following bombardment of the infectious construct pC-35STMVR into *N. tabacum* Samsun *NN* tissue. It was apparent that if future microprojectile bombardment experiments were to succeed the procedure may have to be modified and attention was focused on the target tissue. Successful microprojectile bombardment experiments previously described within this thesis made use of target tissue ~2.25 cm^2, prepared from leaves young leaves, <8 cm long. It was presumed that the undeveloped cuticle on these young leaves would be easy for tungsten particles to penetrate. Furthermore, the leaves should have consisted of closely packed cells with small vacuoles. This should have enhanced the rate at which DNA coated tungsten particles were delivered into the cell nucleus. Yamashita *et al.* (1991) used a 35S-GUS construct to study microprojectile bombardment. They observed a strong correlation, >90%, between cells which expressed GUS and those which received a microprojectile delivered directly into their nucleus. It was thought that cellular damage, caused by heavy bombardment with tungsten microparticles, may have impaired the tissue's ability to effectively induce the HR. It was thought that the use of larger target tissue samples ~9 cm^2 may help overcome this problem. Following the use of larger *N. tabacum* Samsun *NN* tissue samples, lesions and PR-1a protein expression were detected at 4-6 days post bombardment with pC-35STMVR (Figures 7.4; 7.5). The results presented in Figures 7.4 and 7.6 suggested that the development of lesions may have been influenced by the age of target leaf tissue. It has been previously reported that the age of leaves/plants can influence *N* and *N'* gene-mediated resistance to TMV (Goodman and Novacky, 1994; Culver and Dawson, 1991). Microprojectile bombardment experiments, using pC-35STMVR and transgenic *N. tabacum*

Samsun *NN* PR-1a-GUS tissue, were conducted to investigate this further. It was concluded that mature leaf tissue (prepared from leaves >15 cm long) was most suited to the microprojectile bombardment experiments. It is important to remember that many complex biochemical interactions occur as part of the *N* gene mediated HR (Section 1.9.1). It is thought that salicylic acid signals the expression of the PR proteins (Ohshima *et al.*, 1990; Yalpani *et al.*, 1991 Uknes *et al.*, 1993). It is therefore interesting to note that young leaf tissue soaked in salicylic acid gave rise to higher levels of PR-1a promoter expression than mature leaf tissue (Ohshima *et al.*, 1990). Following the microprojectile bombardment of C-35STMVR, lesions were small compared with those photographed in Figure 7.1 and concentric rings of necrosis were not visible. This can be explained by the fact that bombarded tissue samples could only be incubated for 4 days post bombardment without deterioration. It is normal for lesions to appear at 2-3 days post manual inoculation of a *N* gene host plant with TMV particles, and to continue to expand until 8-10 days post inoculation (Deom *et al.*, 1991). The lesions induced following the inoculation of detached leaf tissue were also much darker than those observed following the manual inoculation of an intact plant (compare Figures 7.7 and 7.26 with Figure 7.1). This may be due to the increased production of phenolic compounds in detached necrotising tissue.

It was decided that large tissue samples 9 cm^2 may be more suited to visual observations and histochemical staining and that smaller samples 2.25 cm^2 may be more suited to quantitative fluorimetric analysis. The infectious replication and replicase constructs, and also a control plasmid, were bombarded independently into 9 cm^2 *N. tabacum* Samsun *NN* and transgenic *N. tabacum* Samsun *NN* PR-1a-GUS leaf samples. Lesions only developed on tissue bombarded with the infectious construct, pC-35STMVR (Figures 7.4; 7.12). Upon histochemical staining at 4 days post bombardment, tissue surrounding the lesions developed a dark blue pigment (Figure 7.12). The lesions tended to form in a ring ~1.5 cm in diameter, with fewer lesions in the centre of the ring. As previously suggested, this may be due to the HR being impaired by extensive tissue damage at the centre of target tissue. Alternatively, it is possible that the microprojectile bombardment procedure, in particular the design of the stop plate, directed the majority of tungsten particles towards the edge of the observed ring. Leaf samples bombarded with either the replication construct pCU-XM, replicase construct pTRepTerm or control plasmid pBS SK^- developed some light blue pigment following histochemical staining (Figure 12). This was in a ring ~1.5 cm in diameter, and in some cases was more concentrated at the outer edge. It has been suggested that, particularly in the case of pBS SK^- bombarded tissue, the pigment reflected stress induced PR-1a promoter activity (Section 7.2.3.4). The transgenic *N. tabacum* Samsun *NN* PR-1a-GUS (line 8) plants were selected for their ability to

express high levels of GUS following infection by TMV. Stress induced expression of GUS resulting from the microprojectile bombardment procedure had not been investigated. This could have arisen from cutting the tissue, putting it in a vacuum, penetration with high velocity tungsten particles, and prolonged post bombardment incubation. Stress induced PR-1a protein activity resulting from cutting tissue had been reported by Ohshima *et al.* (1990).

The lack of lesions on tissue bombarded with either pCU-XM or pTRepTerm was not unexpected. Transcripts produced by the constructs would have been incapable of moving out of bombarded cells. It has been reported that lesions failed to develop following the manual inoculation of movement deficient TMV mutants on to host plants expressing the *N* gene (Meshi *et al.*, 1988; Holt and Beachy, 1991). It is possible that the viral elicitor needed to be expressed in a number of adjacent cells, possibly to boost levels above a threshold not attainable in a single cell. The ability of a pathogen to multiply in a cluster of adjoining cells poses an obvious threat to a plant. It is also possible that the HR induced death of single cells may have been too small to visualise or distinguish from damage induced by the inoculation procedure. It was hoped that histochemical staining and/or fluorimetric analysis of bombarded tissue may have detected elevated levels of PR-1a promoter activity in tissue bombarded with either pCU-XM or pTRepTerm. Unfortunately no activities higher than the background induced by bombardment with pBS SK$^-$ were observed (Section 7.2.3.4). By studying the histochemically stained tissue photographed in Figure 7.12, two observations were made i) each individual bombardment was unique and led to highly variable levels of PR-1a promoter activity ii) any differences in PR-1a promoter activity induced by pCU-XM, pTRepTerm and pBS SK$^-$ were very slight. As a result, it is doubtful whether further quantitative experiments would contribute accurate information to the visual observations already made.

Plant transformation provided important information confirming suggestions that the TMV encoded coat protein was the viral elicitor of the *N'* resistance gene (Section 1.9.5; Culver and Dawson, 1991; Pfitzner and Pfitzner, 1992). It was thought that similar experiments may help identify the viral elicitor of the *N* gene. Following co-cultivation with *Agrobacterium* harbouring the infectious construct from pB-35STMV, *N. tabacum* Samsun *NN* leaf discs either remained green, developed black/dark brown necrosis or areas of light brown necrosis (Figures 7.13; 7.14). RNA dot blots revealed that while the green areas remained virus free, TMV was detectable in the necrotic tissue. The appearance of necrosis suggested that TMV infections, induced by the infectious construct from pB-35STMV, were eliciting the *N* gene-mediated HR. The occurence of leaf discs with both green and light brown areas suggested that the

HR was successfully containing the virus. It is unknown why some leaf discs developed the black necrosis. It is possible that it was due to rapid infection, resulting from the successful transformation of many cells in the same leaf disc. This infection would have taken place while leaf discs were recovering from the stresses induced by the transformation and tissue culture procedures. The binary control construct pMKC 6 should not have induced the HR following integration into the genome of *N. tabacum* Samsun *NN* cells. As a result leaf discs co-cultivated with *Agrobacterium* harbouring pMKC 6 remained healthy and developed high numbers of calli and shoots. Only 13 shoots were grown into plants for analysis, as it was judged that this was sufficient to demonstrate that the transformation procedure had been successful. Over 50 % of these regenerated plants expressed high levels of GUS (Table 7.5).

The binary replication construct pBU-XM and replicase construct pBTRepTerm should have been able to express the TMV encoded replicase proteins following integration into the plant genome. If these proteins are responsible for eliciting the *N* gene the HR should have been elicited in transgenic T_0 plants. It is possible that the ability to replicate, via the formation of an active replication complex, is required to elicit the *N* gene. In this case only transgenic plants expressing the replication construct from pBU-XM should have induced the HR. RNA transcribed by pBU-XM and pBTRepTerm should have been incapable of moving from cell-to-cell. As a result the occurrence of necrosis throughout leaf disc explants was not expected. Instead it was thought that induction of the HR may result in symptoms similar to those described by either Culver and Dawson (1991) or Pfitzner and Pfitzner (1992). Both reports describe transforming host tissue expressing the *N'* gene with elicitor 35S-coat protein constructs. Following the transformation of *N. sylvestris* Culver and Dawson (1991) observed that plants regenerated at a slower rate than normally observed. Necrosis and chlorosis started to appear on the leaves of regenerated plants following their transfer to soil. In some cases the necrosis spread to vascular tissue and resulted in death of the plant. Pfitzner and Pfitzner (1992) used *Agrobacterium* to transform *N. tabacum* Samsun EN. They observed reduced callus formation. When callus did form it developed necrosis and in some cases died.

Following the co-cultivation of *N. tabacum* Samsun *NN* leaf discs with *Agrobacterium* harbouring the replication construct from pBU-XM very little callus was observed. Although necrosis on this callus was not obvious, only five shoots developed and only three of these developed kanamycin resistant roots. The three regenerated line M plants were shown to be transgenic (Figure 7.17) and developed very slowly. These results are similar to those reported by Culver and Dawson (1991) and therefore suggest that the replication construct from pBU-XM may be capable of

eliciting the *N* gene. Following transfer to soil, only one of the regenerated plants, M2, developed necrosis which spread to the vascular system and resulted in death of the plant. The delay in the onset of this necrosis could be explained by the HR being impaired in young tissue (as suggested by Culver and Dawson. 1991). However no delay was observed following the co-cultivation of explants with pB-35STMV. Alternatively a threshold level of elicitor may have been required before onset of the HR. We were unable to detect the TMV-U1 126 kDa replicase protein following western immunoblot analysis of samples prepared from the plant M1 (Figure 7.18). Furthermore, the results presented in Section 6.2.3.2 suggest that RNA transcribed from pBU-XM does not replicate and produce coat protein as efficiently as wild type TMV RNA. It is possible that the ability of the HR to function may have been impaired in plants M1 and M3 and this could be associated with the very slow development of these plants. This theory is supported by the inoculation of a transgenic leaf detached from plant M1 with purified TMV particles (Figure 7.25). Although necrosis developed, the normal defined lesions associated with TMV infections on *N* gene host plants were not observed. Instead the virus appeared to spread rapidly throughout the leaf. The result suggested that an impaired HR was causing extensive cellular necrosis and chlorosis, yet was failing to contain the infection. It is interesting to note that additional mechanisms of resistance previously observed in transgenic plants expressing parts of the TMV genome (Section 1.9.6) did not appear to be effective.

The co-cultivation of *N. tabacum* Samsun *NN* leaf discs with *Agrobacterium* harbouring the replicase construct from pBTRepTerm resulted in the development of both callus and shoots. However, only seven of the shoots (out of >18) developed kanamycin resistant roots. All seven of these line R regenerated plants appeared to contain the stably integrated insert (Figure 7.17). The construct had previously been used to transform *N. tabacum* SR1 explants (Section 6.2.3) and resulted in the regeneration of 17 kanamycin resistant line B plants. The numbers of regenerated plants cannot be directly compared as explants were prepared from two different cultivars of *N. tabacum*, and there may have been slight variations in the experimental techniques. The transgenic *N. tabacum* Samsun *NN* line R plants developed at the same rate as plants transformed with the control construct from pMKC 6. The plants appeared to be healthy and demonstrated none of the visual symptoms associated with TMV infection, either in wild type or *N* gene host plants. Western immunoblot analysis did not detect TMV encoded 126 kDa protein in the line R plants. It is possible that the regenerated plants were expressing low levels of these proteins. It should also be noted that the technique also failed to detect the 126 kDa protein in regenerated *N. tabacum* SR1 line B plants which had been transformed with the same replicase construct (Section 6.2.3.2). The inability to detect PR-1 proteins in line M and line R plants was

not unexpected (Figure 7.20), as the proteins are associated with successful induction of the HR. The only evidence that the replicase construct may have interfered with the plant's ability to express the HR came from the manual inoculation of detached leaves with purified TMV particles (Figures 7.23 and 7.24). The lesions that appeared on the detached, inoculated leaves of line R plants were much lighter than those on line G plants expressing the control construct from pMKC 6. In addition, areas of severe yellowing were observed. We were unable to determine how effectively the HR was containing TMV in the areas of necrosis.

The results of microprojectile bombardment experiments, presented within this Chapter, provided evidence that the infectious construct pC-35STMVR was acting in the way expected in host tissue expressing the *N* gene. However the experiments did not help identify the TMV encoded elicitor of the *N* gene. Plant transformation experiments again confirmed that pB-35STMV was acting in the way expected. At the same time they indicated that the replication construct may have been capable of eliciting the *N* gene-mediated HR. In summary, very interesting transgenic plants have been generated which clearly require further analysis. The results may confirm the previous suggestion that the replicase proteins are the TMV encoded elicitors of the *N* gene (Padgett and Beachy, 1993). At the same time other factors may be important, such as the formation of an active replication complex, accumulation of the elicitor above a threshold level or movement of the elicitor into neighbouring cells. It is hoped that further analysis may provide significant information on the HR and the way in which it is elicited by TMV.

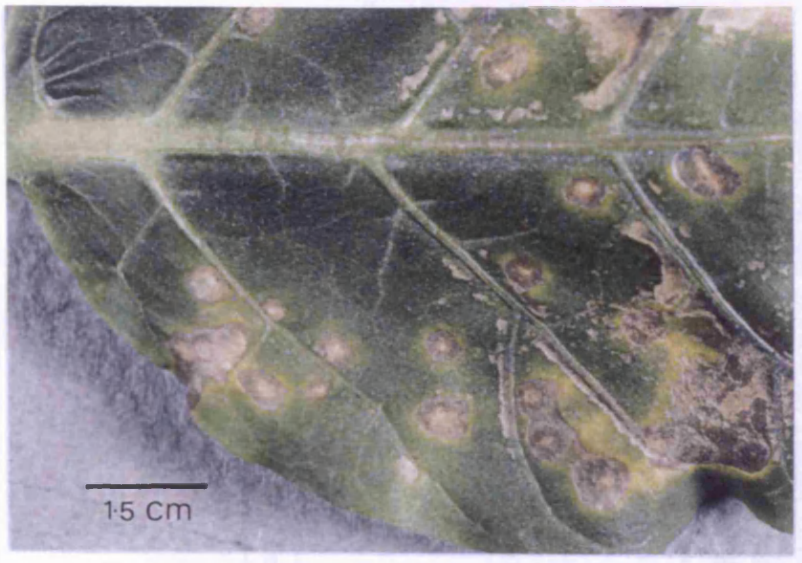


Figure 7.1 Typical TMV Induced Lesions. A mature *N. tabacum* Samsun *NN* leaf, >15 cm long, was manually inoculated with ~0.5 µg of purified TMV-U1 particles. The leaf remained attached to the plant prior to photography at 14 days post inoculation.

Figure 7.2 Results of Preliminary Microprojectile Bombardments. *N. tabacum* Samsun *NN* tissue samples, ~2.25 cm^2, were prepared from leaves <8 cm long. Symptoms were photographed at 8 days post bombardment with pC-35STMVR (A); pU/mcs (B); tungsten particles with out plasmid DNA (C).

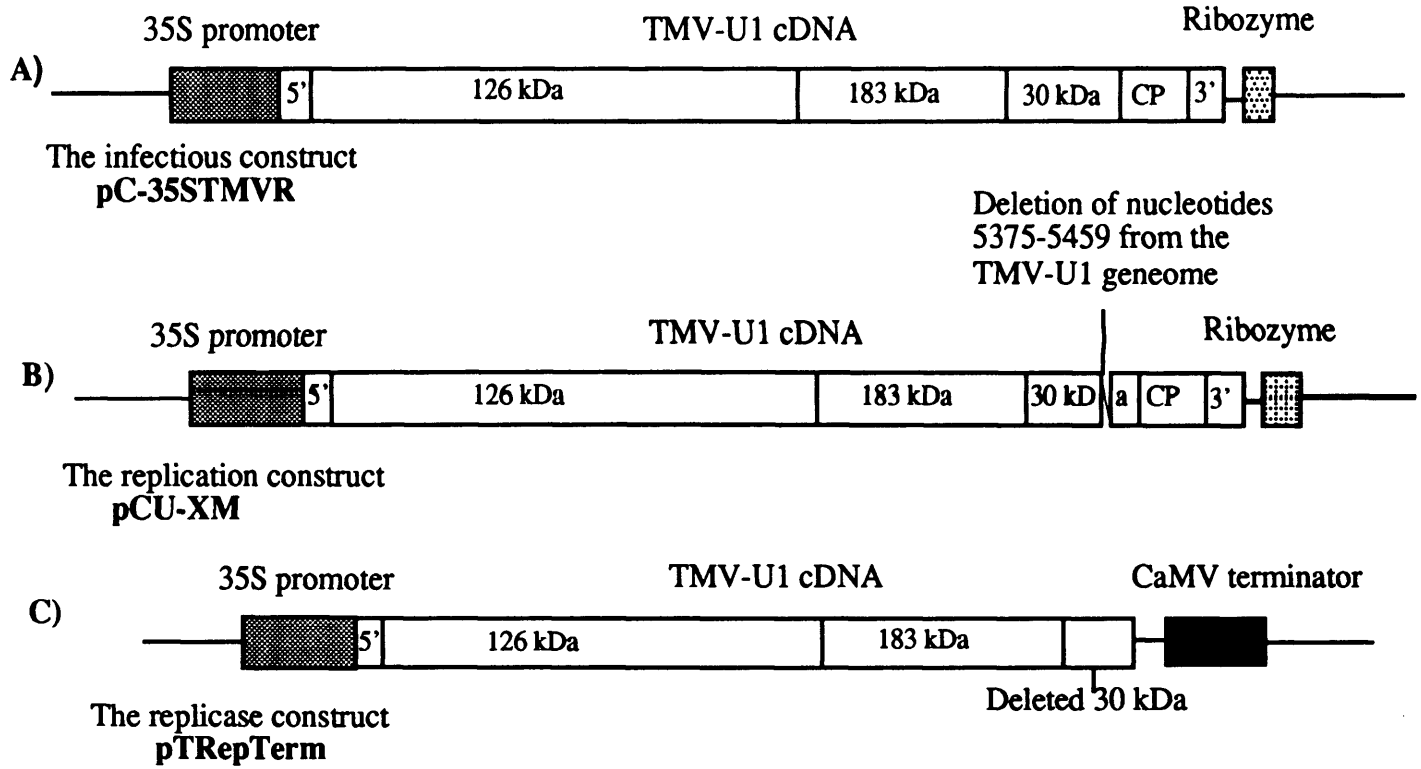


Figure 7.3 Diagrams of pC-35STMVR, pCU-XM and pTRepTerm. The infectious construct pC-35STMVR (A) replication construct pCU-XM (B) and replicase construct pTRepTerm (C) were all in the plasmid vector pCDNA II. The binary constructs pB-35STMV, pBU-XM and pBTRepTerm were essentially the same, with the exception that pB-35STMV no longer included a ribozyme sequence. The binary infectious and replicase constructs were cloned into the vector pBin 19, the binary replication construct was cloned into the vector pPZP111. As the constructs have been described in previous chapters, details on construction have not been provided.

A) pC-35STMVR

B) pCU-XM

C) pTRepTerm

D) pBS SK-

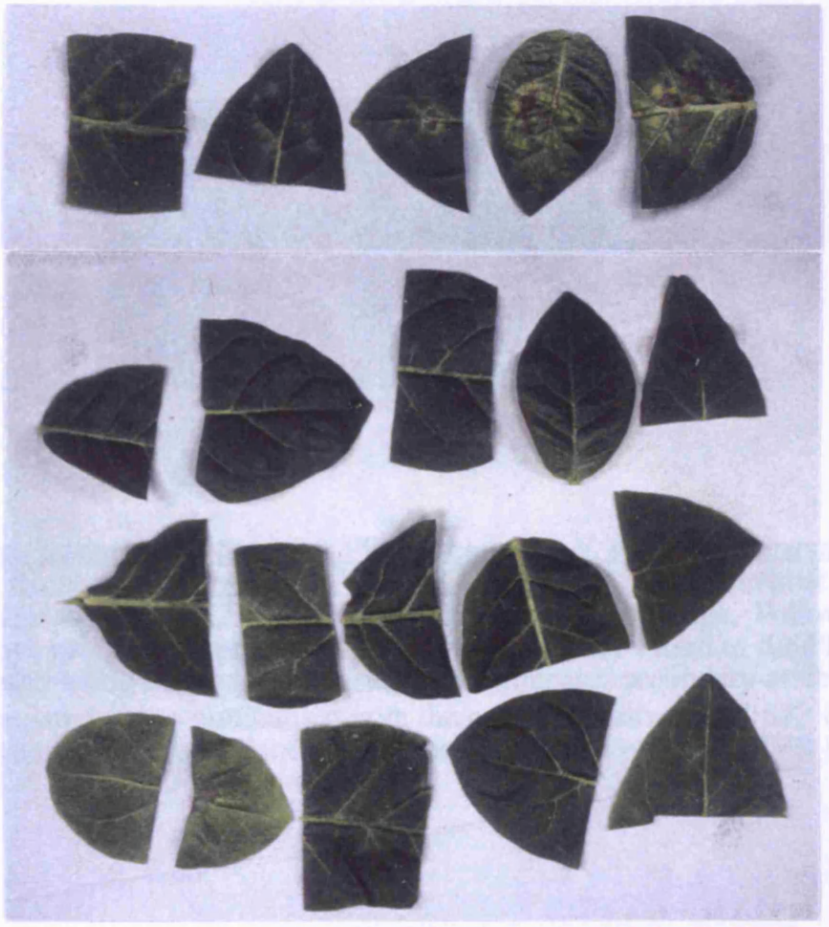


Figure 7.4 Symptoms Induced on *N. tabacum* Samsun *NN* Leaf Tissue Following Bombardment with TMV-based Constructs. Tissue samples ~9 cm^2 were prepared from leaves of mixed age. Samples were photographed at 4 days post bombardment with the infectious construct pC-35STMVR (A); replication construct pCU-XM (B); replicase construct pTRepTerm (C); control plasmid pBS SK^- (D).

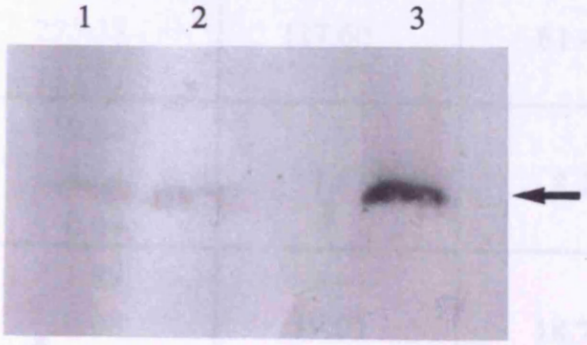


Figure 7.5 Western Immunoblot Showing PR-1 Proteins in *N. tabacum* Samsun *NN* Tissue Following Bombardment with TMV-based Constructs. Each construct was bombarded into 6 leaf samples ~2.25 cm^2 taken from leaves of mixed age. Following incubation for 6 days protein was prepared, probed with antibody raised to the PR-1a protein and visualised using an alkaline phosphatase conjugated secondary antibody. Protein samples prepared tissue bombarded with the control construct pBS SK^- (lane 1); replicase construct pTRepTerm (lane 2); infectious construct pC-35STMVR (lane 3). Position of the ~15 kDa PR-1 proteins (arrow).

Figure 7.6 TMV Induced Symptoms on a Young *N. tabacum* Samsun *NN* Leaf. A detached leaf <6 cm long was photographed at 7 days post manual inoculation with ~5 µg of purified TMV-U1 particles.

Construct	pMol 4MU min^{-1} $mg\ protein^{-1}$	Mean	Standard deviation from mean
pC-35STMVR	24.17 223.12 135.51	127.60	81.41
pCU-XM	19.22 4.34 0.04	7.87	8.22
pTRepTerm	1.89 10.08 45.09	19.02	18.73
pBS SK^-	4.76 203.83 6.27	71.62	93.48

Table 7.1 Quantitative Comparison of PR-1a Promoter Activities in Tissue Bombardment with TMV-based Constructs. The infectious construct pC-35STMVR, replication construct pCU-XM, replicase construct pTRepTerm and a control plasmid pBS SK^- were bombarded into *N. tabacum* Samsun *NN* PR-1a-GUS tissue. Fluorimetric GUS analysis was carried out at 4 days post bombardment of each construct into three individual tissue samples ~2.25 cm^2 prepared from leaves of mixed age. The construct pRTS2-LUC was used as an internal control. Luciferase assays were carried out at 24 hour post bombardment and resulted in activities ranging from 116090-521220 LU, where pCU-XM < pC-35STMVR < pBS SK^-< pTRepTerm.

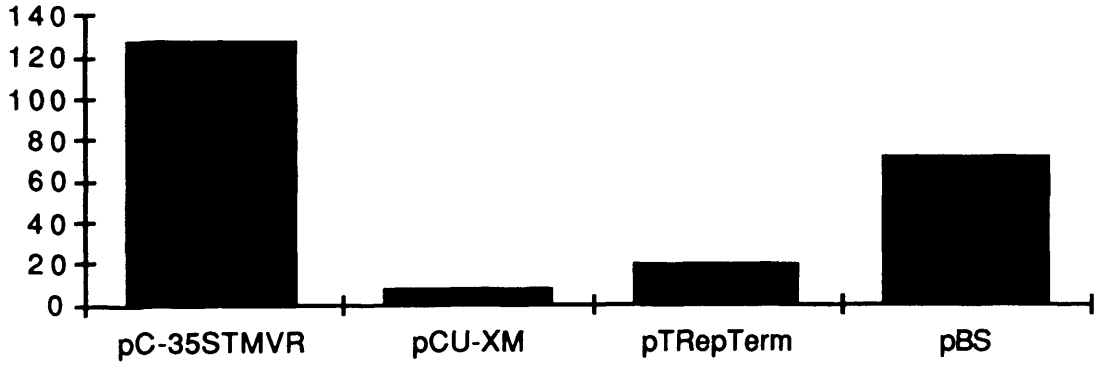


Figure 7.7 Bar Chart Showing Data Presented in Table 7.1. PR-1a promoter activities were compared following the bombardment of *N. tabacum* Samsun *NN* PR-1a-GUS tissue with TMV-based constructs. Fluorimetric GUS analysis was carried out at 4 days post bombardment of each construct into three individual tissue samples 2.25 cm^2 prepared from leaves of mixed age. Y axis equals pMol 4MU min^{-1} $mg\ protein^{-1}$.

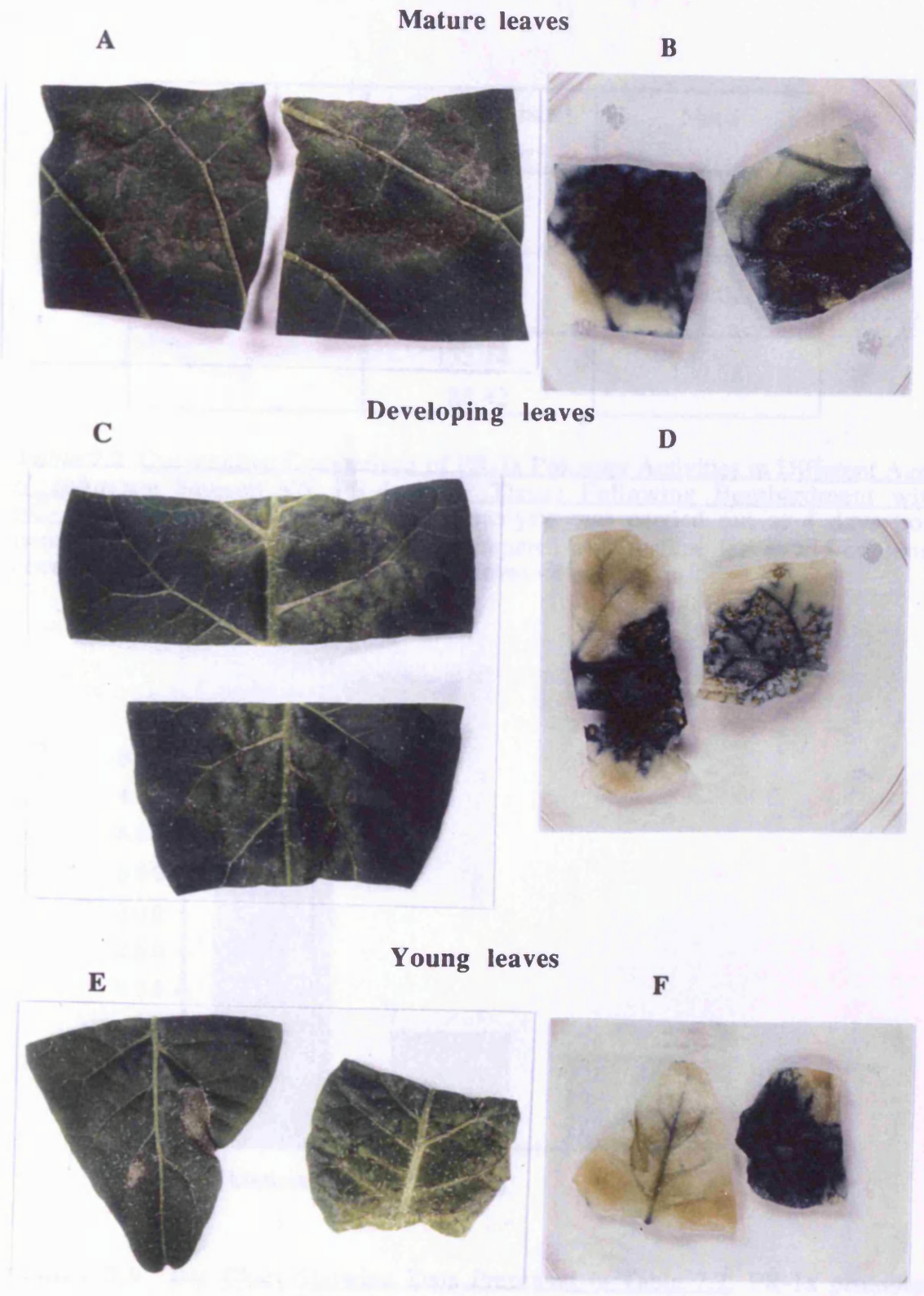


Figure 7.8 PR-1a Promoter Activity in Different Aged *N. tabacum* Samsun *NN* PR-1a-GUS Tissue Following Bombardment with pC-35STMVR. Tissue samples ~9 cm^2 were bombarded with the infectious construct pC-35STMVR. At 4 days post bombardment samples were photographed (A, C, E) and histochemically stained to indicate PR-1a promoter activity (B, D, F). Target tissue taken from mature leaves >15 cm long (A, B); developing leaves 10-12 cm long (C, D); young leaves <8 cm long (E, F).

Type of Leaf	pMol 4MU min^{-1} $mg\ protein^{-1}$	Mean
Mature	548.97 395.63	472.30
Developing	159.25 136.78	148.01
Young	152.74 88.42	120.58

Table 7.2 Quantitative Comparison of PR-1a Promoter Activities in Different Aged *N. tabacum* Samsun *NN* PR-1a-GUS Tissue Following Bombardment with pC-35STMVR. Fluorimetric GUS analysis was carried out at 4 days post bombardment into two samples ~9 cm^2 prepared from mature leaves >15 cm long; developing leaves 10-12 cm long; young leaves <8 cm long.

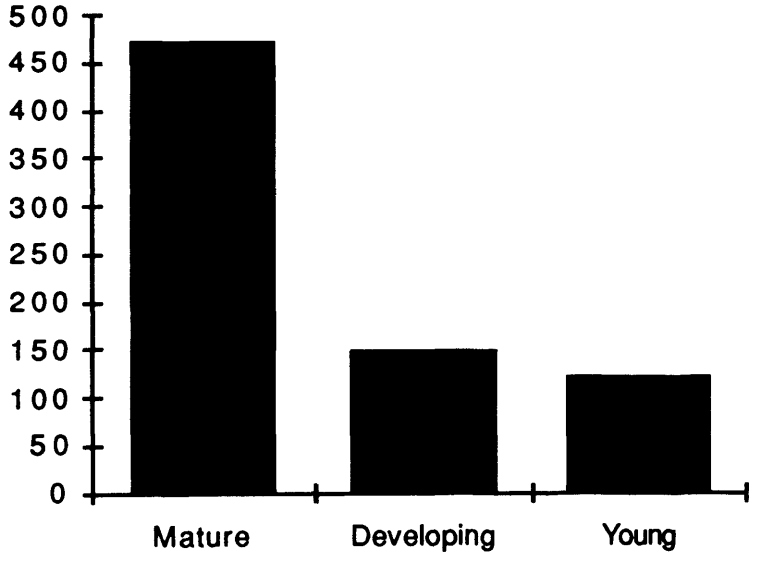


Figure 7.9 Bar Chart Showing Data Presented in Table 7.2. PR-1a promoter activities were compared following the bombardment of different aged *N. tabacum* Samsun *NN* PR-1a-GUS leaf tissue with pC-35STMVR. Fluorimetric GUS analysis was carried out at 4 days post bombardment into two samples ~9 cm^2 prepared from mature leaves >15 cm long; developing leaves 10-12 cm long; young leaves <8 cm long. Y axis equals pMol 4MU min^{-1} mg $protein^{-1}$.

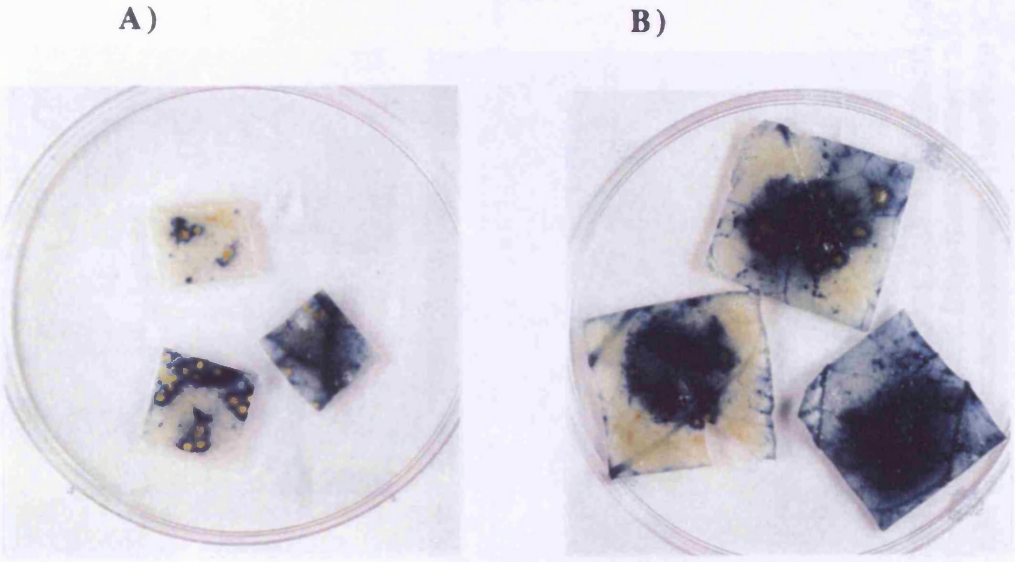


Figure 7.10 PR-1a Promoter Activity in Different Sized *N. tabacum* Samsun *NN* PR-1a-GUS Tissue Following Bombardment with pC-35STMVR. Target leaf tissue was prepared from mature leaves >15 cm long and bombarded with the infectious construct pC-35STMVR. Histochemical staining was carried out at 4 days post bombardment into target tissue samples ~2.25 cm^2 (A); ~9 cm^2 (B).

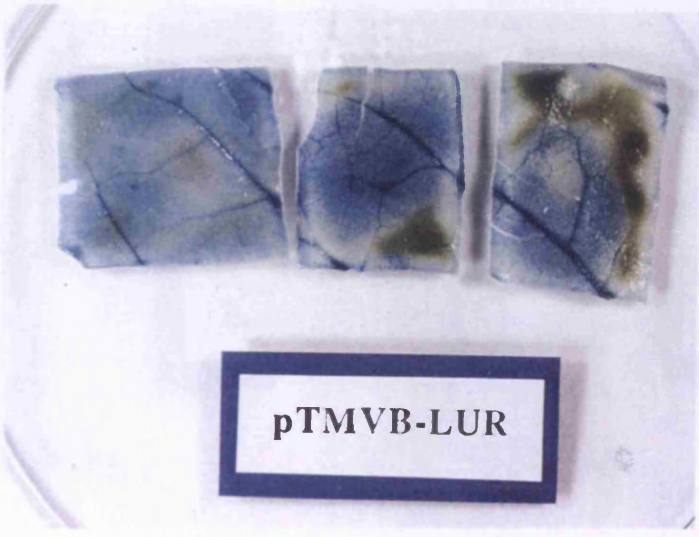


Figure 7.11 PR-1a Promoter Activity in *N. tabacum* Samsun *NN* PR-1a-GUS Tissue Following Bombardment with pTMVB-LUR. Histochemical staining was carried out at 4 days post bombardment into tissue samples ~9 cm^2 prepared from mature leaves >15 cm long.

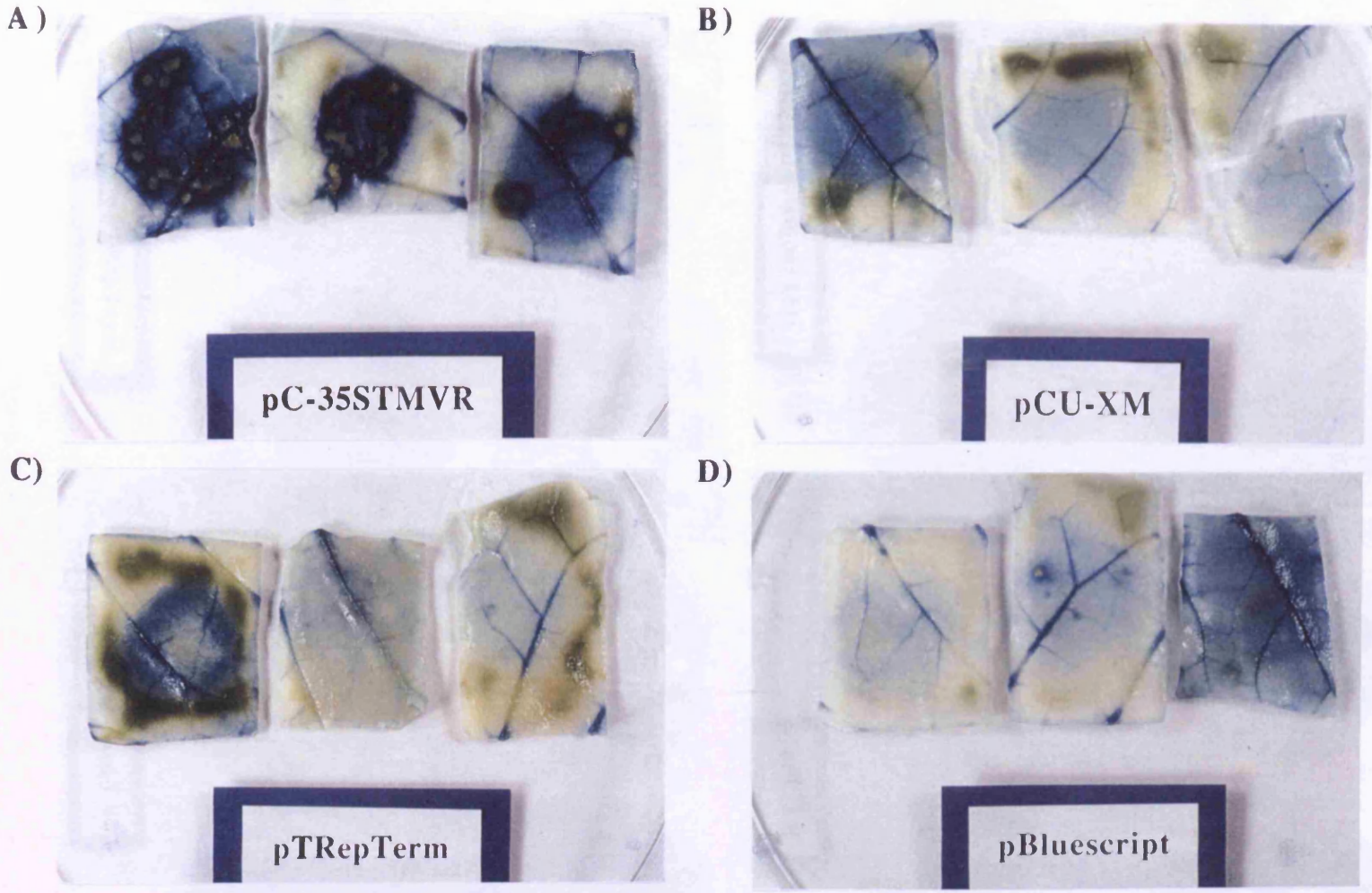


Figure 7.12 PR-1a Promoter Activity in *N. tabacum* Samsun *NN* PR-1a-GUS Tissue Following Bombardment with TMV-based Constructs. Histochemical staining was carried out at 4 days post bombardment into target tissue samples ~9 cm^2 prepared from mature leaves >15 cm long. Tissue bombarded with the infectious construct pC-35STMVR (A); replication construct pCU-XM (B); replicase construct pTRepTerm (C); control plasmid pBS SK^- (D).

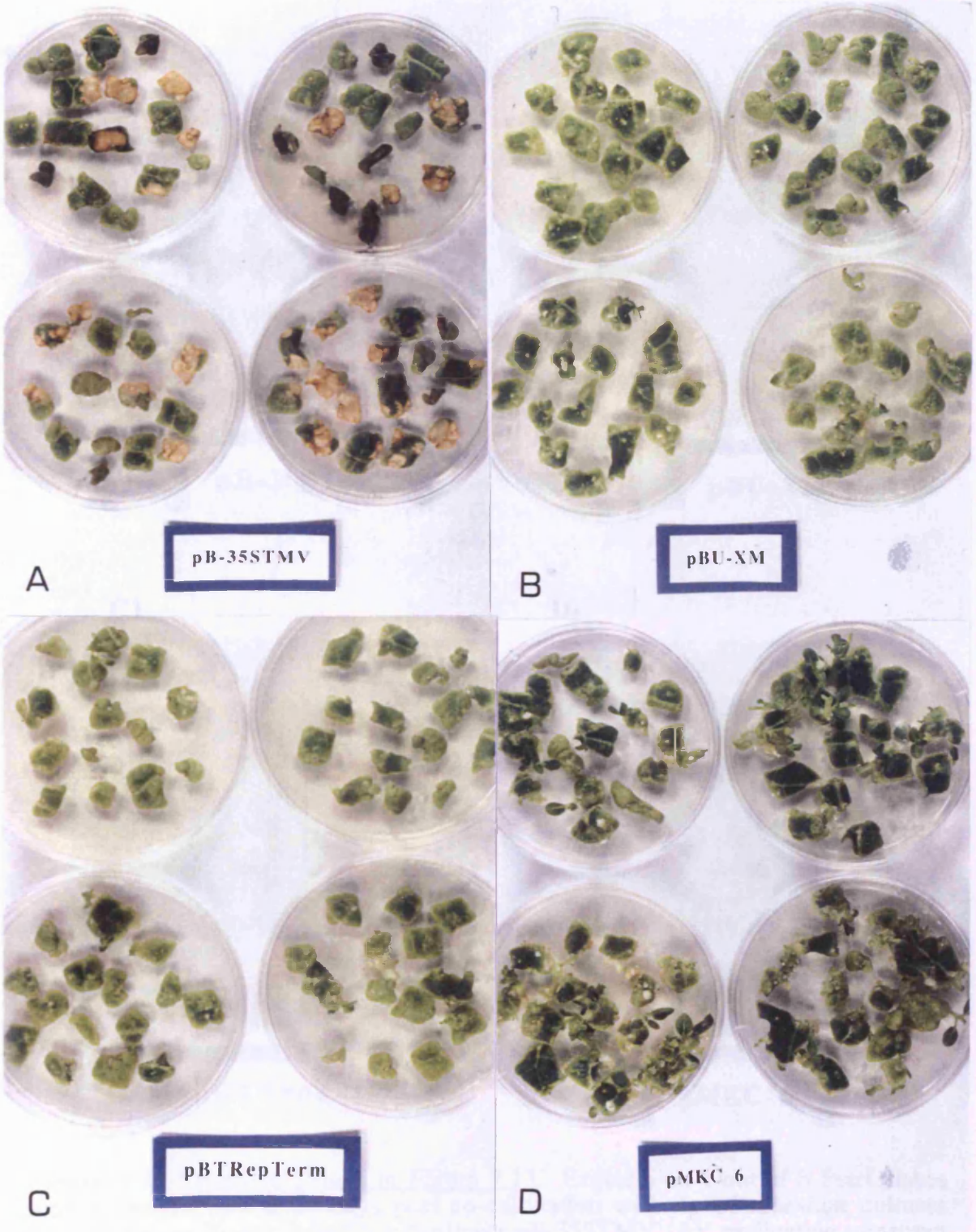


Figure 7.13 *N. tabacum* Samsun *NN* Explants Following Transformation with TMV-based Constructs. Explants on 4 out of 8 Petri dishes were photographed at 28 days post co-cultivation with *Agrobacterium* cultures harbouring the binary infectious construct pB-35STMV (A); replication construct pBU-XM (B); replicase construct pBTRepTerm (C); control construct pMKC 6 (D).

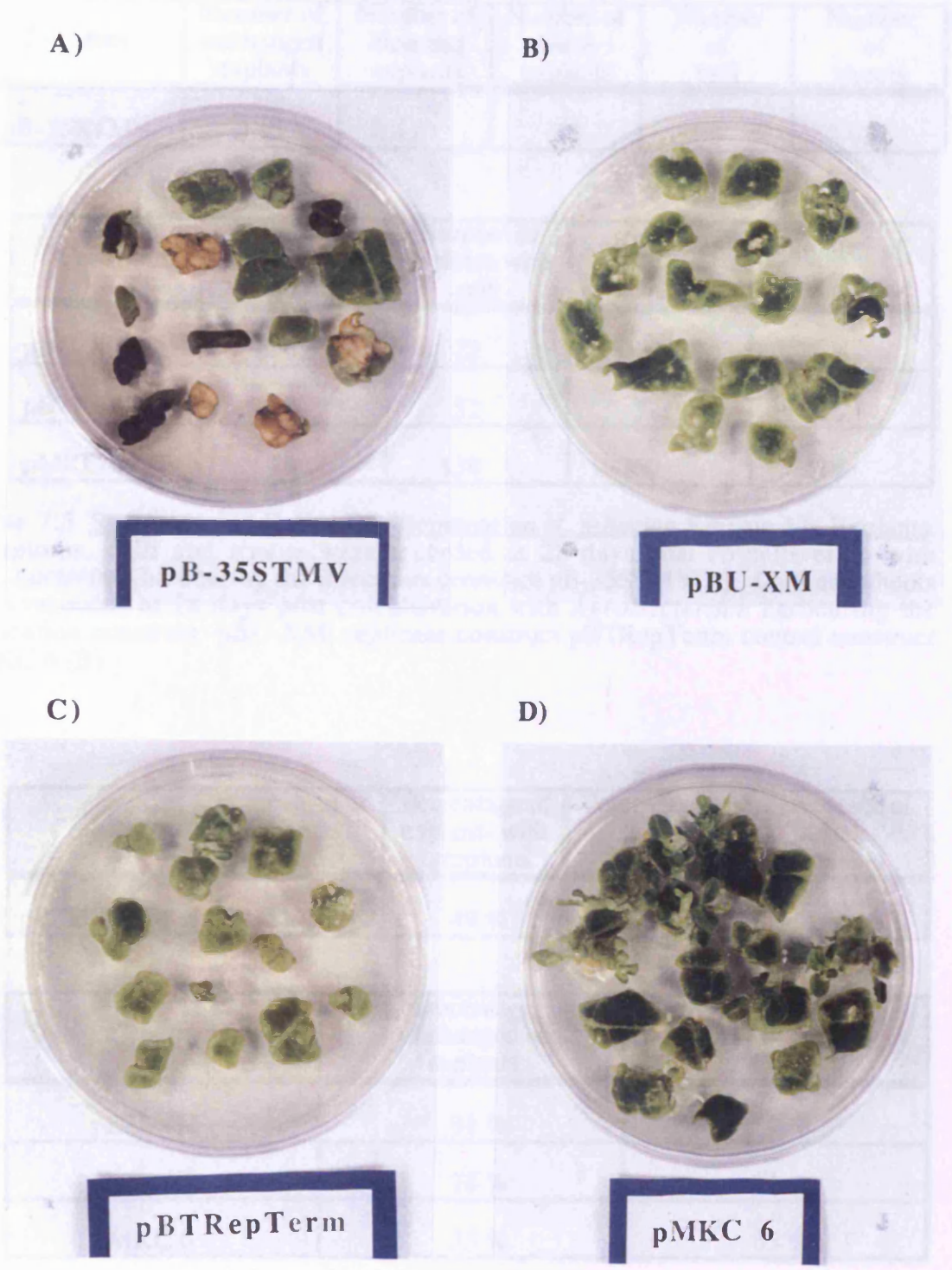


Figure 7.14 Detail of Panels in Figure 7.13. Explants on 1 out of 8 Petri dishes were photographed at 28 days post co-cultivation with *Agrobacterium* cultures harbouring the binary infectious construct pB-35STMV (A); replication construct pBU-XM (B); replicase construct pBTRepTerm (C); control construct pMKC 6 (D).

A)

Construct	Number of unchanged explants	Number of bleached explants	Number of black explants	Number of calli	Number of shoots
pB-35STMV	130	110	15	3	3

B)

Construct	Number of unchanged explants	Number of explants with calli	Number of calli	Number of shoots
pBU-XM	220	22	29	2
pBTRepTerm	188	52	72	18
pMKC 6	74	138	>250	115

Table 7.3 Symptom and Callus Development on *N. tabacum* Samsun *NN* Explants. Symptoms, calli and shoots were recorded at 28 days post co-cultivation with *Agrobacterium* harbouring the infectious construct pB-35STMV(A). Calli and shoots were recorded at 28 days post co-cultivation with *Agrobacterium* harbouring the replication construct pBU-XM; replicase construct pBTRepTerm; control construct pMKC 6 (B)

A)

Construct	Percentage of unchanged explants	Percentage of explants with symptoms	Percentage of bleached explants	Percentage of black explants
pB-35STMV	51 %	49 %	43 %	6 %

B)

Construct	Percentage unchanged of explants	Percentage of explants with calli
pBU-XM	91 %	9 %
pBTRepTerm	78 %	22 %
pMKC 6	35 %	65 %

Table 7.4 Data Presented in Table 7.3: Expressed as Percentages. Explants displaying symptoms and calli have been expressed as a percentage of the total number of explants. Symptoms were recorded at 28 days post co-cultivation with *Agrobacterium* harbouring the infectious construct pB-35STMV (A). Calli were recorded at 28 days post co-cultivation with *Agrobacterium* harbouring either the replication construct pBU-XM, replicase construct pBTRepTerm or control construct pMKC 6.

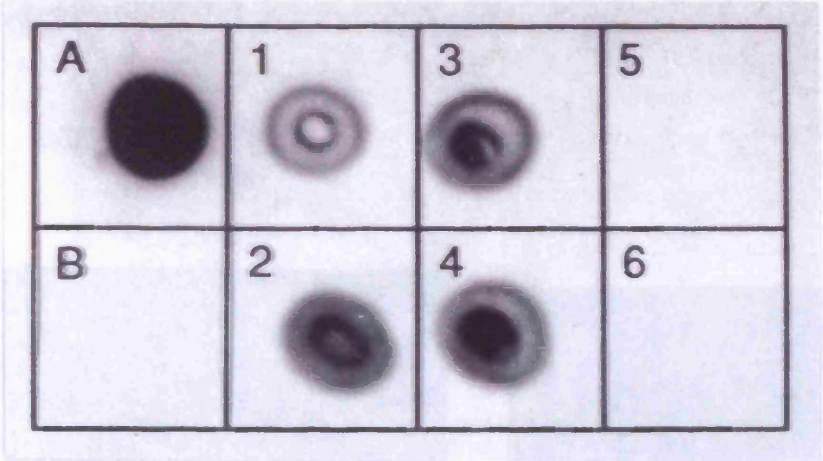


Figure 7.15 RNA Dot Blot to Detect TMV in *N. tabacum* Samsun *NN* Leaf Discs Following Co-cultivation with pB-35STMV. The blot was probed with an ~3.6 kb *Hind* III fragment isolated from pC-35STMVR and exposed to X-ray film for 24 hours. Purified TMV particles (A); dH_2O (B). Sap prepared from light brown leaf discs (1, 2); black leaf discs (3, 4); green leaf discs (5, 6).

Figure 7.16 Variable Phenotypes on *N. tabacum* Samsun *NN* Line M Plants. Plants M3 (A) and M2 (B) had been transformed with the replication construct from pBU-XM. The photograph was taken shortly after the plants were transferred to soil. Developing necrosis lead to the death of plant M2.

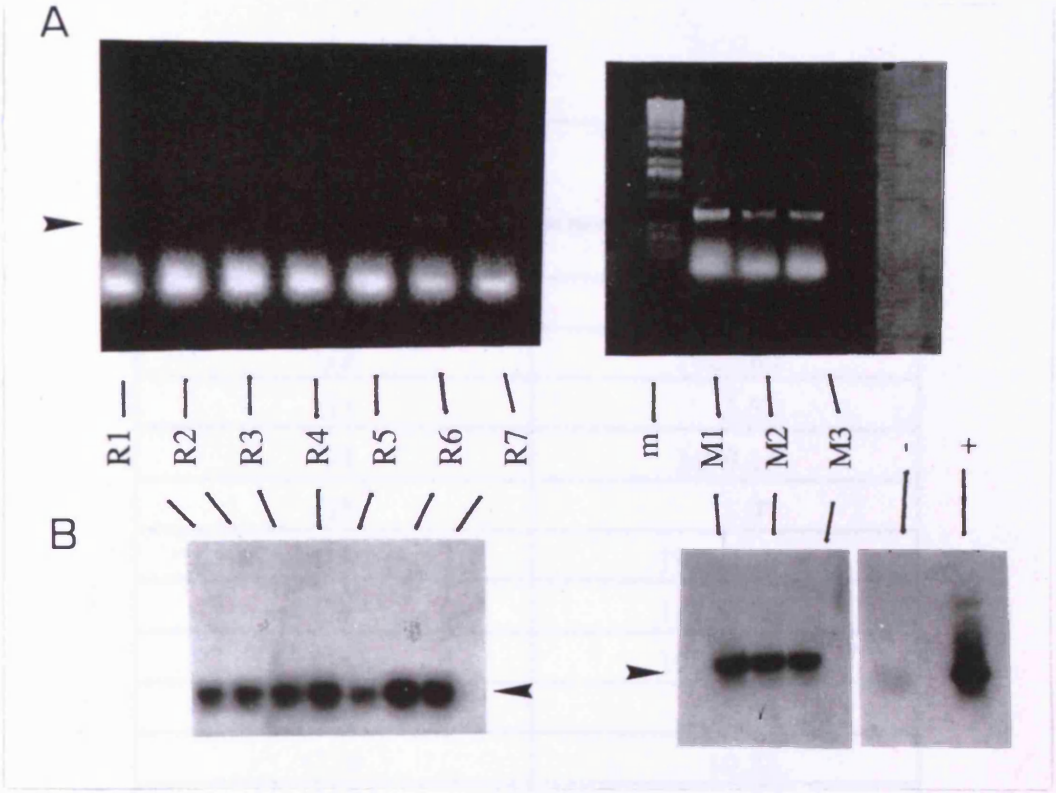


Figure 7.17 PCR Analysis of *N. tabacum* Samsun *NN* Line M and Line R Plants. The primers 5'REP and 3'REP were used to amplify a 735 bp fragment from line M plants, transformed with the replication construct from pBU-XM; line R plants, transformed with the replicase construct from pBTRepTerm. Agarose gels showing PCR products (A). Southern blots probed with an ~700 bp *Sst* II, *Bam*H I fragment isolated from pC-35STMVR (B). The blots were exposed to X-ray film for 4 hours. Molecular weight markers (m), PCR using the construct pC-35STMVR (+); genomic DNA extracted from an untransformed *N. tabacum* SR1 plant (-). PCR amplified fragments are indicated by the arrows.

Table 7.5 Fluorimetric GUS Analysis of *N. tabacum* Samsun *NN* Line G Plants. The ability to express GUS was used to indicate that the regenerated plants had been stably transformed with the control 35S-GUS construct from pMKC 6.

Plant	pMol 4MU min^{-1} $mg\ protein^{-1}$
Untransformed control	0.68
G1	24330.39
G2	1121.80
G3	2.58
G4	3199.15
G5	1.97
G6	19149.90
G7	11350.80
G8	10562.40
G9	0.90
G10	10.33
G11	3.98
G12	19.37
G13	14921.24

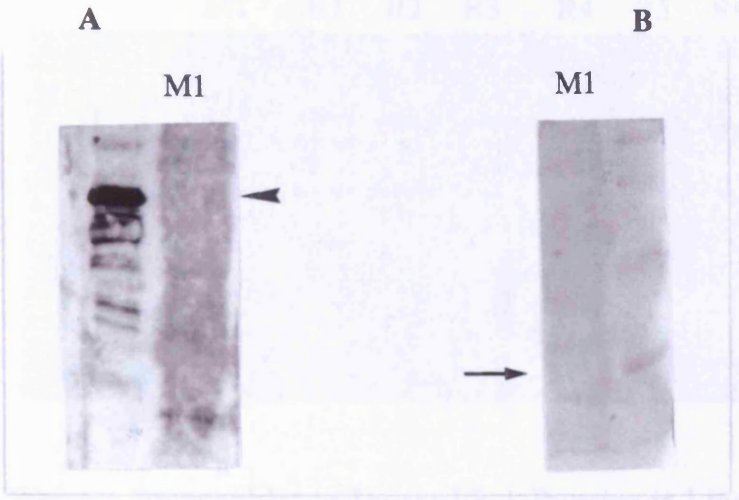


Figure 7.18 Western Immunoblots to Detect TMV Encoded Proteins in the Plant M1. The regenerated plant should have been expressing the replication construct from pBU-XM. Protein was probed with antibody raised to the TMV-U1 126 kDa replicase protein (A) and 17.5 kDa coat protein (B). Visualisation was carried out using an alkaline phosphatase conjugated secondary antibody. Size of 116 kDa molecular weight marker (➤), 24 kDa molecular weight marker (⟶). Positive and negative control samples, probed simultaneously, have been presented in Figures 6.6 and 6.7.

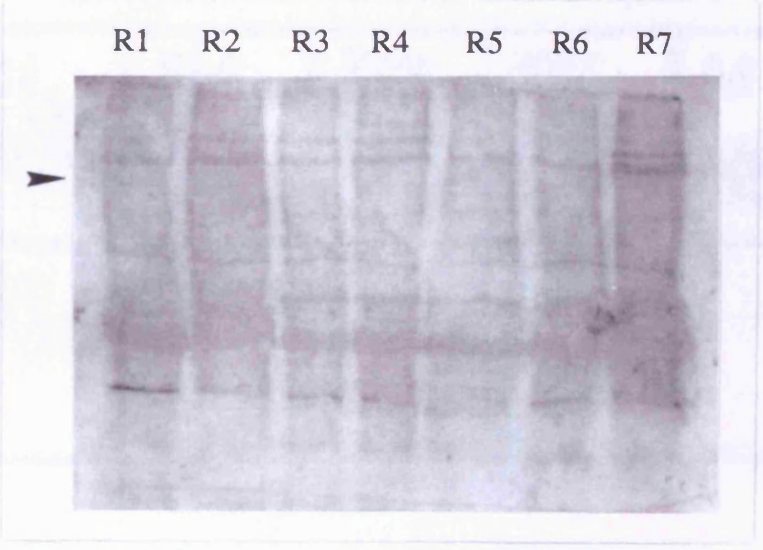


Figure 7.19 Western Immunoblot to Detect the TMV 126 kDa Protein in Line R Plants. The regenerated plants should have been expressing the replicase construct from pBTRepTerm. Approximately equal concentrations of protein were probed with antibody raised to the TMV-U1 126 kDa replicase protein and visualised using an alkaline phosphatase conjugated secondary antibody. The 116 kDa molecular weight marker is indicated by the arrow. Positive and negative control samples, probed simultaneously, are presented in Figure 6.6.

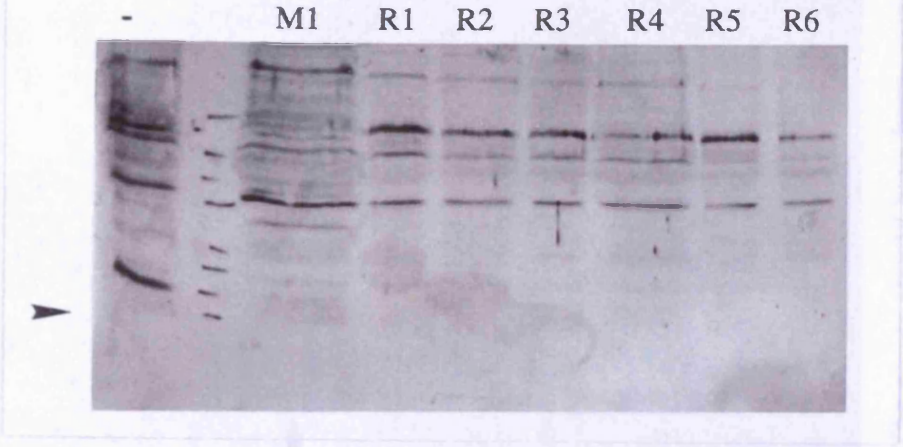


Figure 7.20 Western Immunoblot to Detect PR-1 Proteins in Line M and Line R Plants. Approximately equal concentrations of protein were probed with antibody raised to the PR-1a protein and visualised using an alkaline phosphatase conjugated secondary antibody. Protein sample prepared from an untransformed *N. tabacum* SR1 plant (-). The 24 kDa molecular weight marker is indicated by the arrow.

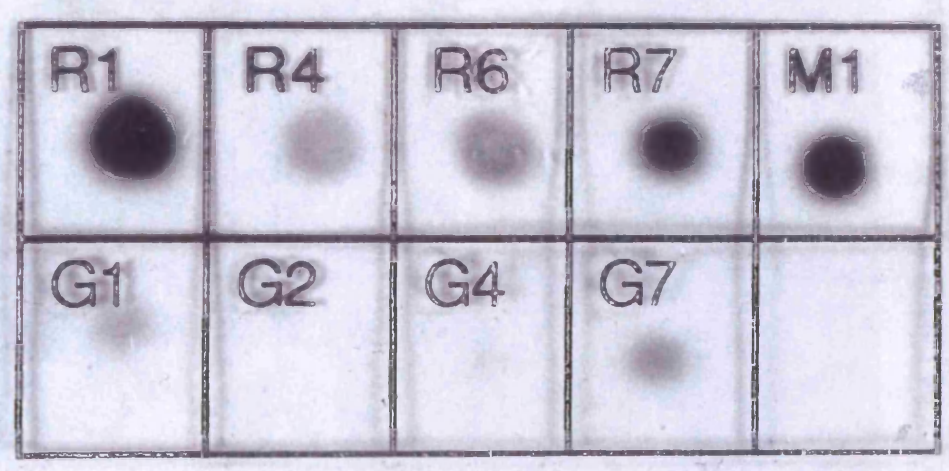


Figure 7.21 RNA Dot Blot to Detect TMV RNA in the TMV Inoculated Leaves of Line G, R and M Plants. Plant lines had been transformed with the 35S-GUS construct from pMKC 6, replicase construct from pBTRepTerm and replication construct from pBU-XM, respectively. Detached leaves were manually inoculated with ~0.5 µg of purified TMV particles. Extracts were prepared from leaf tissue at 5 days post inoculation. The blot was probed with an ~3.6 kb *Hind* III fragment isolated from pC-35STMVR and exposed to X-ray film for 24 hours.

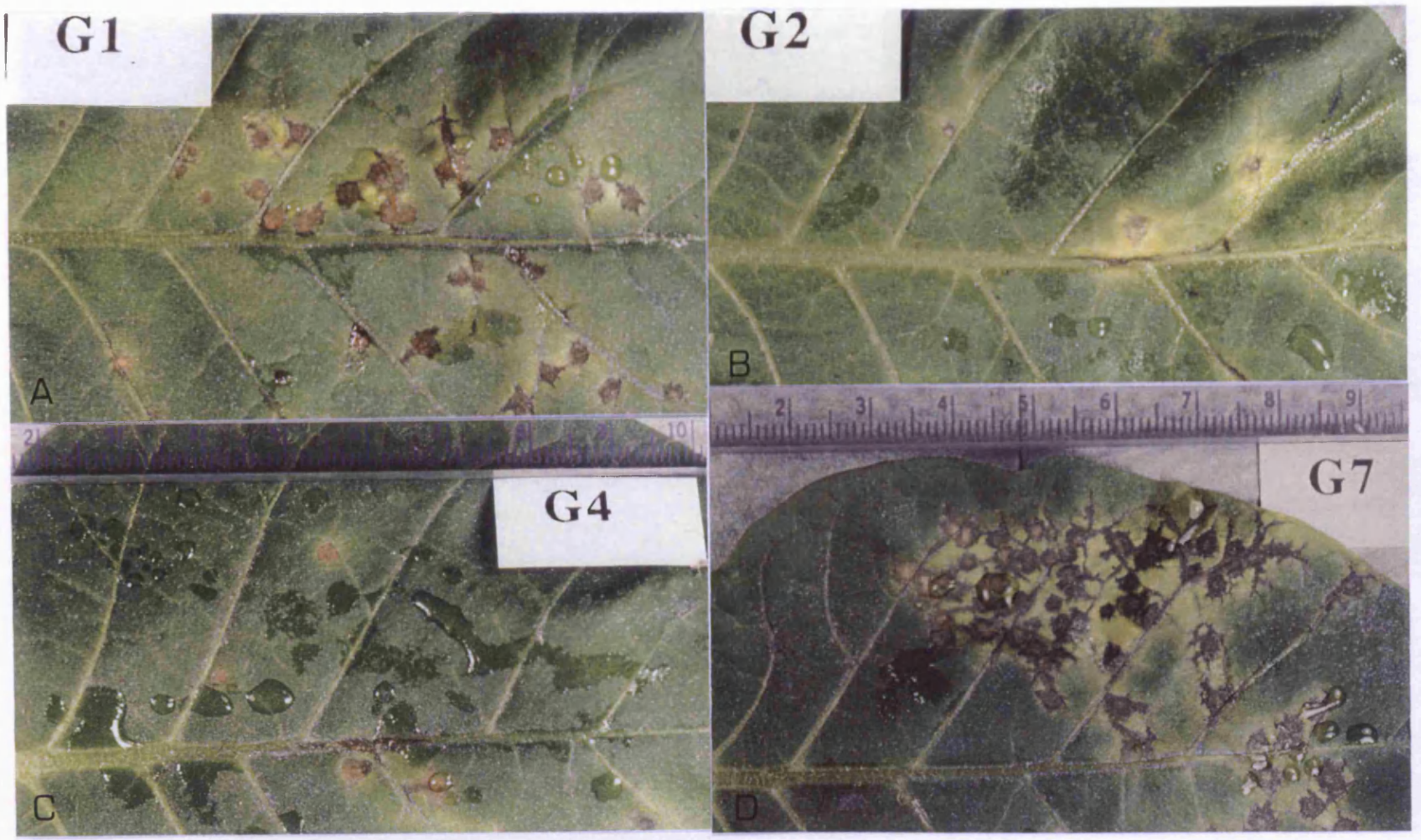


Figure 7.22 TMV-Induced Symptoms on Leaves from *N. tabacum* Samsun *NN* Line G Plants. The plants should have been expressing the control 35S-GUS construct from pMKC 6. Leaves were detached from plant lines G1 (A); G2 (B); G4 (C); G7 (D). Photographs were taken at 5 days post manual inoculation with ~0.5 μg of purified TMV particles. All photographs are the same magnification, ruler represents cm.

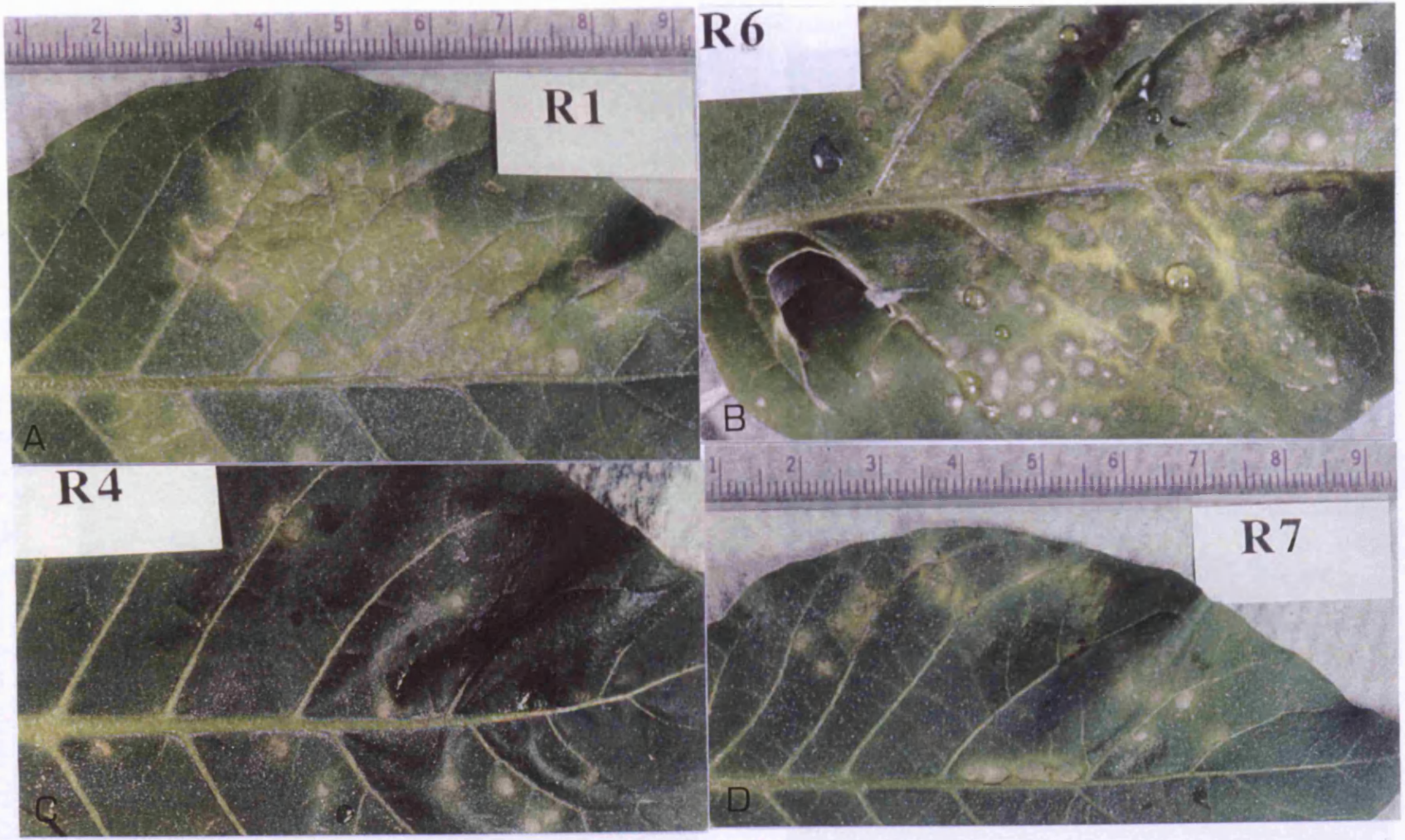
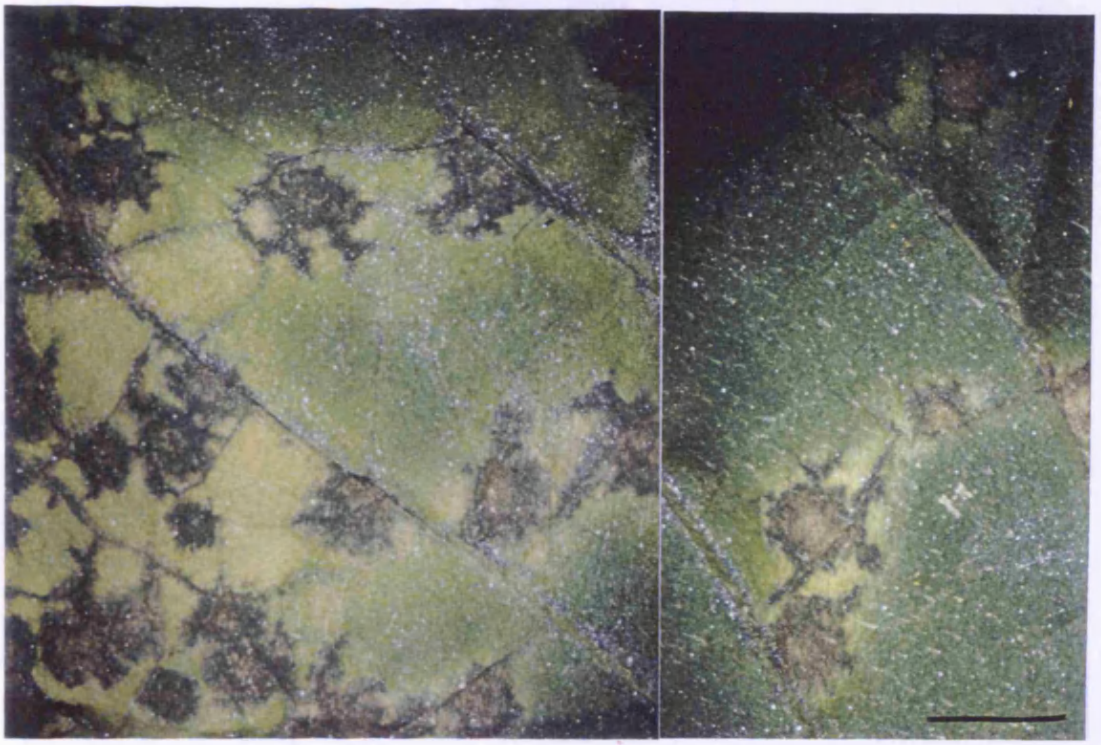


Figure 7.23 TMV-Induced Symptoms on Leaves from *N. tabacum* Samsun *NN* Line R Plants. The plants should have been expressing the replicase construct from pBTRepTerm. Leaves were detached from plant lines R1 (A); R6 (B); R4(C); R7 (D). Photographs were taken at 5 days post manual inoculation with ~0.5 µg of purified TMV particles. All photographs are the same magnification, ruler represents cm.

A) G7



B) R6

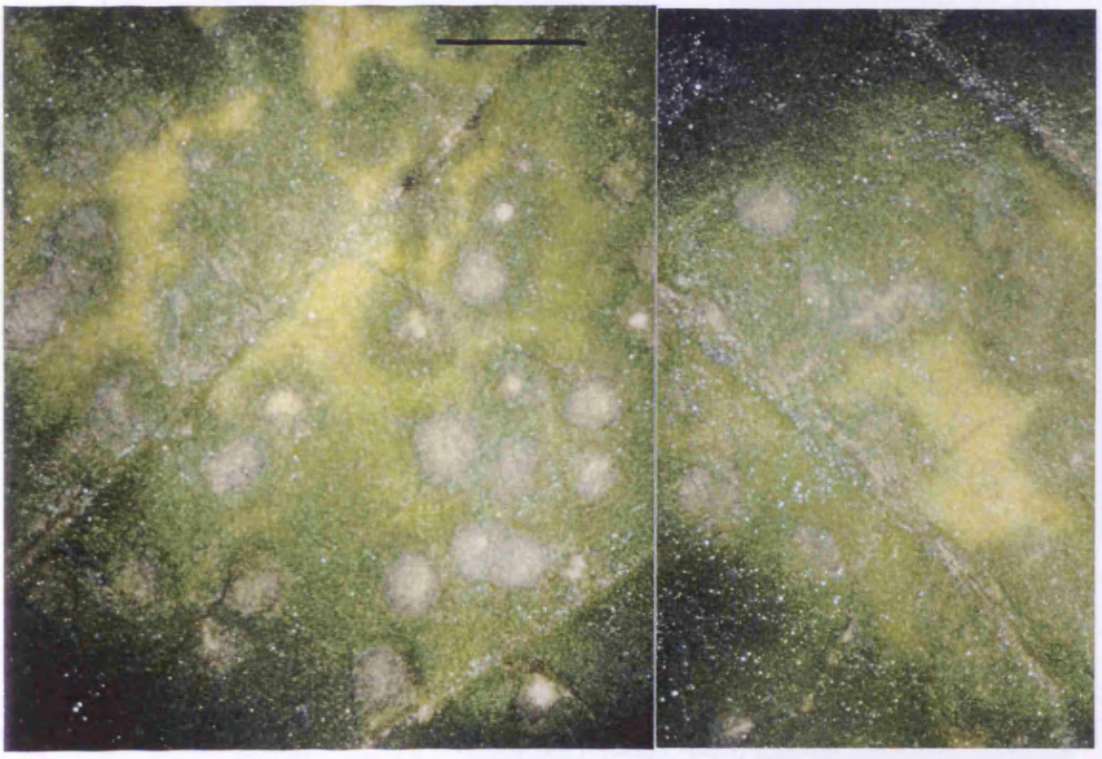


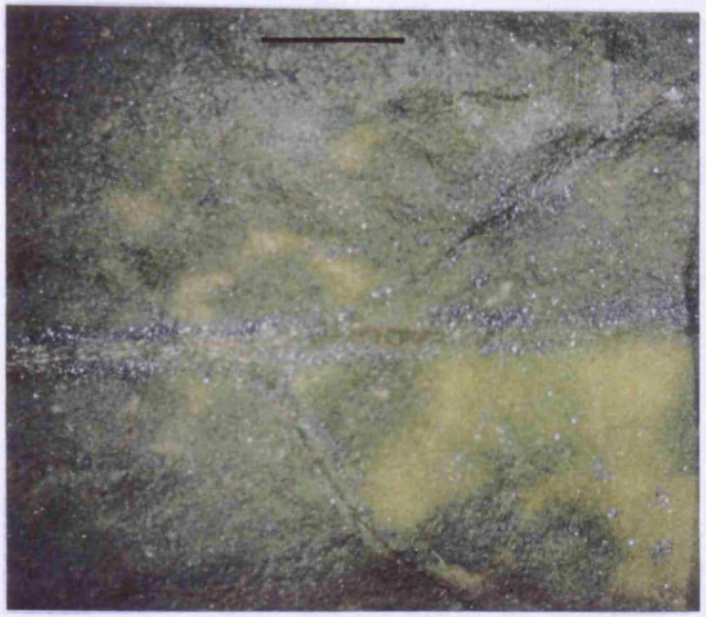
Figure 7.24 Magnified TMV-Induced Symptoms on Leaves From Plants G7 and R6 (see Figures 7.22 and 7.23, respectively). The leaves from plants G7 (A) and R6 (B) were manually inoculated with ~0.5 µg of purified TMV particles and incubated for 5 days prior to photography. Bars represent ~0.5 cm.

Figure 7.25 TMV-Induced Symptoms on a Leaf from *N. tabacum* Samsun *NN* Plant M1. The plant should have been expressing the replication construct from pBU-XM. The leaf was detached from the plant and inoculated with ~0.5 µg of purified TMV particles. Following incubation for 5 days symptoms were photographed (A), the ruler represents cm. Symptoms magnified ~ 3x (B and C), Bar represents ~0.5 cm.

A)

B)

C)



CHAPTER 8

FINAL DISCUSSION AND CONCLUSIONS

The study of phenotypic mutants can provide important information concerning the way in which genes interact with other genetically controlled and/or environmental factors. The ability to precisely manipulate nucleic acids facilitates this research. As a result, valuable information can be gained and existing theories can be tested. The ability to generate full length cDNA clones revolutionised the study of plant viruses with RNA genomes. The ability to transcribe the cDNA clones *in vitro* by fusing them to bacterial promoters (section 1.4.2) meant that the effects of mutations could be studied. However, if experiments were to be conducted using intact plants it was essential that RNA transcribed by the clones maintained the ability to replicate and move from initially infected cells. It should be noted that limited investigations were possible using RNA that was no longer infectious. For example, studies could be conducted in isolated protoplasts. Recent reports of infectious viral cDNA clones which can be transcribed *in vivo* by the 35S promoter from CaMV are encouraging. Such constructs are suitable for both transient assay procedures and stable integration into the genomes of plants. This thesis demonstrates the importance of 35S-cDNA constructs. Using an infectious 35S-cDNA clone of TMV-U1 a number of useful transient and plant transformation experiments have been conducted. As a result, information has been gained concerning infectivity, the production of TMV-based expression vectors and host resistance to TMV.

In order to justify the application of the 35S-TMV constructs described in sections 3.2.1 and 3.2.2, it was important to determine their ability to infect plants known to host TMV. It was essential that the constructs were suitable for plant transformation experiments. In addition, their application in protoplast and microprojectile bombardment experiments was considered to be favourable. Although only one experiment was conducted using protoplasts the result was encouraging (Section 3.2.4). The infectious construct p35STMVR was able to induce a TMV infection, measured by the production of infectious sap at 24 hours post inoculation. We are confident that our ability to isolate and inoculate protoplasts would have improved with practise. As a result, we anticipate that it should be possible to detect TMV encoded proteins within 24 hours (Siegel *et al.*, 1978; Watanabe *et al.*, 1984). Protoplasts allow the synchronous inoculation of large numbers of cells. As a result, they are particularly useful for transient assays using virus-based constructs which are no longer capable of cell-to-cell movement. Although they were not widely used in experiments detailed within this thesis they may prove to be useful for further studies. For example, transient experiments using the replication and replicase constructs could have been conducted in protoplasts prepared from *L. esculentum* GCR 26 (+/+) and *L. esculentum* GCR 237 (*Tm-1/Tm-1*) plants. Such experiments would complement the attempted *L. esculentum* transformation experiment detailed in Chapter 6. Both

p35STMVR and pC-35STMVR were highly infectious if bombarded into *N. tabacum* SR1 and *L. esculentum* GCR 26 (+/+) tissue (Section 3.2.5). TMV RNA and coat protein was easily detected at 4 days post bombardment and infectious sap could be harvested within 24 hours. It was anticipated that microprojectile bombardment may be useful for studying TMV-based constructs which maintained the ability to move from cell-to-cell or efficiently expressed a marker gene. However, it was thought that the technique may be unsuitable for directly monitoring the production of TMV encoded proteins if they were confined to initially bombarded cells. The binary infectious construct pB-35STMV proved to be suitable for plant transformation experiments. As a result, it was anticipated that agroinoculation experiments (Grimsley *et al.*, 1986) could also have been attempted. Although time consuming, plant transformation may have been one of the most suitable techniques for studying constructs in large numbers of cells over a period of time. However, it should be noted that gene expression and regulation may be significantly affected by the continued expression of constructs. This could be due to the saturation of cells with particular proteins or the absence of certain proteins involved with regulation. As a result, plant transformation may only complement transient assays or inoculation experiments. For curiosity, manual inoculation experiments were conducted (Section 3.2.3). Many of these were unsuccessful and possible reasons have been discussed in Section 3.3. It was thought that the inability of cDNA constructs to reach the cell nucleus may be the reason for failure of the manual inoculation experiments. If true, it was thought that the solution may be hard to establish. Having concluded that p35STMVR was infectious and suitable for the investigations detailed in Chapters 4-7, it was decided to cease manual inoculation experiments. It was possible to either inoculate protoplasts or bombard tissue in order to produce infectious sap. If necessary, this could have been used for manual inoculation experiments. Furthermore, we were prepared to view p35STMVR as a complement to existing cDNA clones of TMV, fused to bacterial promoters (Section 1.4.2), instead of a replacement.

Chapters 4 and 5 describe the generation and analysis of four different replication-marker constructs (Figures 4.6; 4.7; 5.6; 5.7). It was hoped that they could be used as part of a transposon tagging initiative aimed at isolating the *Tm-1* resistance gene (Section 1.10). In addition, they should have been suitable for use in many experiments where levels of TMV replication required monitoring. Each construct contained the luciferase marker gene in place of the coat protein ORF. Upon the inoculation of tissue known to host TMV, the replicase proteins should have been translated from the 5' end of construct RNA (Section 1.6.3) transcribed by the 35S promoter. The proteins should have joined with host proteins and construct RNA to form an active replicase-complex (Section 1.6.2). This complex should have been able

to recognise the TMV 3' untranslated region (Section 1.6.4) and as a result produce (-)-sense construct RNA. The replication complex should then have recognised the coat protein subgenomic promoter within (-)- sense RNA (Section 1.6.6). As a result, a subgenomic RNA should have been generated, from which the luciferase protein should have been translated.

The replication-marker constructs pU-SLU and pU-SLLUTR were cloned into three different plasmid vectors to suit experimental needs (Section 4.2.2). The constructs were analysed via transient microprojectile bombardment and plant transformation experiments. The replication-marker constructs pTMVLUR and pTMVB-LUR have been described in Sections 5.2.1 and 5.2.2. They were analysed using transient microprojectile bombardment experiments. The results of all analysis experiments were disappointing. Following bombardment into *N. tabacum* SR1 tissue, none of the constructs expressed levels of luciferase higher than those detected in unbombarded tissue or in tissue which had been bombarded with a control construct pU-S4-L1. This construct lacked the TMV-3' untranslated region. As a result, it should have been incapable of expressing the luciferase gene via the production of subgenomic RNA. T_1 generation *N. tabacum* SR1 plants expressing either the binary control construct pBU-S4-L1, or one of the binary replication-marker constructs pBU-SLU or pBU-SLLUTR, were analysed. Although the levels of luciferase expression were slightly above background it was not possible to distinguish between the three different transgenic plant lines. The construct pU-SLLUTR contained an unintentional deletion within the TMV 3' untranslated region, this could have accounted for its inability to express luciferase. The reasons behind the failure of the remaining replication-marker constructs to express luciferase remains unclear (thorough discussions have been presented in Sections 4.3 and 5.3). Each construct shared similarities with previously described TMV-based expression vectors (Section 1.8). Although the design of each construct could be faulted, no obvious faults were common to all three. The suitability of the luciferase gene has been discussed (Section 5.3). Luciferase assays are very sensitive. Efficient expression of the gene can result in activities $>10^5$ orders of magnitude above background (personal observation). One problem with the luciferase gene may have been its size. It was significantly larger than the majority of marker genes previously use in TMV-based expression vectors (Section 1.8). As a result, it may have been more likely to accumulate lethal mutations. Furthermore the subgenomic promoter and start codon would have been significantly removed from the 3' end of the TMV genome, this may have decreased levels of expression (Section 1.6.8). The instability of the luciferase protein may have been a disadvantage. However, it may have been beneficial for measuring current, as opposed to past, levels of viral replication. Further analysis of plants independently transformed

with the four replication-marker constructs is required to help understand why the constructs failed to express luciferase in the manner intended. Probably the most useful form of analysis would be northern blot analysis as the technique should be able to detect whether full length and subgenomic RNAs were being expressed. Similar analysis could also be carried out to determine the activity of constructs provided by Dr. C. Holt (Section 5.3, final paragraph). The development of replication-marker constructs suitable for plant transformation remains an important objective. However, we believe that the thorough investigation of existing constructs is required before further improvements can be identified and implemented.

The initial objective of experiments detailed in Chapter 6 was to investigate resistance conferred by the *Tm-1* gene (Section 1.9.2). It was thought that levels of TMV RNA and proteins could be compared in *L. esculentum* GCR 26 (+/+) and *L. esculentum* GCR 237 (*Tm-1/Tm-1*)plants following their independent transformation with TMV-based replication and replicase constructs (Figures 6.6.1 and 6.2). Meshi *et al.* (1987) provided evidence which suggested that the *Tm-1* gene conferred resistance by interacting with the TMV encoded replicase proteins. It is possible that significantly lower levels of the proteins may have been detected in *L. esculentum* GCR 237 (*Tm-1/Tm-1*) plants expressing either the replication or replicase constructs, when compared with similarly transformed *L. esculentum* GCR 26 (+/+) plants. This may have indicated that the *Tm-1* gene was capable of destroying the proteins or inhibiting their translation. This would be reflected by a reduction in viral replication and subgenomic RNA production. Alternatively, reduced levels of TMV RNA and proteins may have only been detectable in *L. esculentum* GCR 237 (*Tm-1/Tm-1*) transformed with the replication construct. This may have indicated that the *Tm-1* gene destroyed or inhibited the action of the replicase-complex (Section 1.6.2). As the *L. esculentum* transformation experiments were unsuccessful we were unable to investigate the action of the *Tm-1* gene. The *L. esculentum* transformation protocol has been discussed in Section 6.3. We do not anticipate further problems with the procedure, provided that feeder cells are used during the preincubation and co-cultivation of explants.

The successful independent transformation of the *N. tabacum* SR1 plants with the replication construct from pBU-XM and the replicase construct from pBTRepTerm has been described in Chapter 6. Western immunoblot analysis failed to detect the TMV encoded 126 kDa replicase protein in samples prepared from the transgenic plants. However, the TMV encoded coat protein was detected in samples prepared from plants X5 and X8 (Figure 6.7). For coat protein to be expressed, RNA transcribed by the construct must have been capable of replication and the production of

subgenomic RNAs. However, levels of replication appeared to be lower than those achieved by authentic TMV RNA. This must have been due to the 85 nucleotide deletion contained within the TMV cDNA construct. The deletion was situated within the TMV 30 kDa protein ORF and OAS. The T_0 generation plants X5 and X8 both died prior to setting seed. Although their death cannot definitely attributed to expression of the replication construct (the plants were suffering from an unscheduled whitefly infestation!), it is interesting that they were the only two lines to definitely express coat protein. The remaining regenerated plants were allowed to set seed. It would be interesting to continue investigating the expression of TMV RNA and proteins in T_1 generation line B and line X plants. We have also considered generating further line X plants for analysis. It is possible that having saturated transgenic cells, the replicase proteins were no longer expressed. Furthermore, they may have been stored in a manner which made them hard to detect by western immunoblot analysis. It may be interesting to examine transgenic tissue using an electron microscope to determine whether structures similar to viroplasms or X-bodies (Section 1.6.1) were present.

Having demonstrated that pC-35STMVR was capable of infecting *N. tabacum* SR1 tissue (Chapter 3), it was decided to investigate its ability to elicit the *N* gene-mediated HR. To achieve this the construct was bombarded into *N. tabacum* Samsun *NN* and *N. tabacum* Samsun *NN* PR-1a-GUS tissue. At the same time it was decided to investigate the effect of TMV-based replication and replicase constructs (Figure 7.3) on the same tissue. The microprojectile bombardment technique required optimising to suit our experimental needs. We determined that target tissue samples prepared from recently matured leaves >15 cm long were most suitable for the investigations. Target tissue samples ~9 cm^2 were prepared if symptoms and/or PR-1a promoter activity needed to be visualised. Smaller target tissue samples ~2.25 cm^2 were prepared if the quantitative analysis of PR-1a promoter activity was required. Lesions developed within four day on *N. tabacum* Samsun *NN* tissue following bombardment with pC-35STMVR. It is thought that the TMV encoded replicase proteins may interact with the *N* gene in order to elicit the HR (Padgett and Beachy, 1993). It has also been observed that movement-deficient mutant did not induce visible lesions on tissue expressing the *N* gene (Meshi *et al.*, 1988; Holt and Beachy, 1991). In agreement with this observation, tissue bombarded with either the replication construct, pCU-XM or replicase construct pTRepTerm remained essentially symptomless. RNA transcribed by both constructs would have been incapable of cell-to-cell movement. While induction of the HR could not be visualised it was thought it may be detected if tissue was analysed for PR-1a promoter activity (Section 1.9.1). Unfortunately levels of PR-1a promoter activity above background were not identified. Heitz (1994) demonstrated that, like other acidic PR proteins,

PR-1a expression was localised in the tissue in and immediately surrounding lesions. For the purpose of our experiment, it may have been more appropriate to use plants expressing a GUS construct fused to the promoter of a basic PR protein (we did not have access to such plants). The basic PR proteins (PR-2b, PR-3 and PR-5) tend to be expressed at elevated levels throughout an inoculated leaf (Heitz, 1994).

It was decided to transform the infectious, replication and replicase constructs into *N. tabacum* Samsun *NN* plants. As expected, explants co-cultivated with the infectious construct from pB-35STMV developed necrosis and no transgenic plants were regenerated. Only three line M plants were regenerated. These should have been expressing the replication construct from pBU-XM. One (M2) developed necrosis and died upon transfer to soil (Section 7.2.). The remaining two (M1 and M3) developed slowly and displayed stunted phenotypes, M3 later died. Only plant M1 survived to set seed, these have now been harvested for further analysis. From the results it seems likely that the replication construct was capable of inducing the HR. It is possible that only low levels of the HR elicitor were expressed in the regenerated plant M2. These may have gradually increased as the plant grew leading to induction of the HR. It is also possible that, selection pressures had resulted in the development of plants with an impaired HR. This hypothesis was supported following the inoculation of a leaf from plant M1 with TMV (Figure 7.25). Although necrosis developed on the leaf it was not contained within defined lesions and was accompanied by severe yellowing. We feel that it is essential to conduct further investigations using intact T_1 generation line M1 plants. If we have selected a mutant with a defective HR it is possible that crosses could be performed to separate the mutated locus from the *N* gene. In this case the TMV-based replicase construct may have been a valuable tool, enabling studies of the HR. Line R plants should have been expressing the replicase construct from pBTRepTerm. These plants developed normally, and as a result it could be concluded that the replicase proteins alone were not capable of eliciting the *N* gene-mediated HR. It has been demonstrate the TMV encoded replicase proteins can complement replicase-defective mutants, which do not express the proteins, *in trans* (Ogawa *et al., 1991*; Ogawa *et al.*, 1992) It would be interesting to observe whether necrosis developed if line R plants were inoculated with similar replicase-defective mutants. If necrosis was observed it would indicate that the formation of a replicase complex (Section 1.6.2) is required to elicit the *N* gene-mediated HR. Although the line R plants appeared to develop normally, we remain curious about the uncharacteristic grey lesions which developed on detached leaves, following inoculation with TMV (Figures 7.23; 7.24).

As expected, *N. tabacum* SR1 line U plants, transformed with the infectious construct from pB-35STMVR, developed severe mosaic symptoms. Five additional transgenic *N. tabacum* SR1 plant lines have been described within this thesis. Each line should have been expressing the TMV encoded replicase proteins and line X plants should have been expressing the TMV encoded coat protein. Chlorosis can be hard to observe on *N. tabacum* plants and varied growth conditions did not permit detailed studies. However, it is interesting that none of the transgenic plant lines developed mosaic symptoms. It has been suggested that both the TMV encoded replicase and coat proteins may be responsible for inducing mosaic symptoms (Sections 1.7.1; 1.7.3). Threshold levels of elicitor proteins may not have been expressed in the transgenic plants. However, coat protein was clearly detected in plants X5 and X8. This indicated that reasonably high levels of the replicase proteins must also have been present.

A number of preliminary experiments have been conducted on transgenic *N. tabacum* SR1 and *N. tabacum* Samsun *NN* plant lines aimed at investigating resistance. It was thought that the expression of certain sequences from the TMV genome may confer a resistant phenotype. Investigations were carried out using intact line L, T and C plants and detached leaves from line X and B plants. Many of the results were encouraging and warranted further investigations. Such investigations have been conducted using T_1 generation plants B1, B2, B3, B4, B5, B6 and B8. Six seedlings from each line were inoculated with purified TMV-U1 particles. At 14 days post inoculation, all six B1 plants remained symptomless together with some B2 and B8 plants. The remaining plants developed symptoms similar to those induced by purified TMV-U1 particles. RNA dot blots were used to confirm that inoculated and systemic leaf samples, taken from symptomless plants, remained virus free. The lack of TMV coat protein in line B1 plants was also confirmed using western immunoblot analysis. These results are encouraging, although we are unsure about how they should interpreted. Donson *et al.*, (1993) reported that plants expressing a single truncated replicase protein were resistant to TMV (Section 1.9.6). However, plants expressing intact 126 kDa and 183 kDa replicase proteins remained susceptible to infections. Further investigations are required to determine whether resistant line B plants expressed the TMV encoded replicase proteins. If the replicase construct is capable of conferring resistance in transgenic plants, small lesions may have been expected following the inoculation of *N. tabacum* Samsun *NN* line R plants. However, the lesions which developed following the inoculation of leaves from line R plants were a similar size to those on leaves from control line G plants.

Possible mechanisms of virus-mediated resistance in transgenic plants have recently been discussed by Baulcombe (1996). Mechanisms of RNA mediated protection were considered which resulted in the accumulation of similar mRNAs and viral RNAs being suppressed. It was suggested that suppression may be triggered following high levels of expression of these RNAs. Alternatively suppression may occur following the recognition of certain foreign RNA sequences. The suppression of RNA accumulation could account for resistant phenotypes displayed by the transgenic plants described within this thesis. For example, the resistant line B plant could have been expressing TMV replicase gene RNA above a threshold level. As a reaction to this, the plants may have suppressed the accumulation of the TMV RNA transcribed *in vivo* or introduced by inoculation with virus particles. A threshold level of RNA may be more readily reached in transgenic plants expressing viral RNA capable of replication. Consequently, plants transformed with such constructs may be both resistant to TMV and incapable of expressing RNA and proteins in the manner intended. This may have accounted for i) the low levels of luciferase expressed by line T plants transformed with the replication-marker construct from pBU-SLU ii) the low levels of coat protein expressed by line X plants transformed with the replication construct from pBU-XM.

This thesis has highlighted the use of 35S-TMV constructs for investigating host-virus interactions. In particular, we have concentrated on interactions which result in resistance to TMV. The infectious construct p35STMVR has been used to build many TMV-based constructs. We are satisfied that the suitability of p35STMVR has been adequately demonstrated. It is hoped that analysis of transgenic plant lines expressing replication-marker constructs may identify important information. This may help the design and application of future constructs. A number of other transgenic plant lines have been discussed. Evidence suggests that the generation and analysis of these lines may provide valuable information concerning resistance conferred by the *Tm-1* gene, resistance conferred by the *N* gene, the hypersensitive response to pathogen attack, and resistance conferred by sequences from viral genomes.

The future application of cDNA clones of TMV may provide valuable information aiding our understanding of the virus. While designing the protocols for future experiments it is intriguing to note that some researchers may plan to go further afield, see: "Survival of Tobacco Mosaic Virus in Space" Orlob and Lorenz, (1968) and "Studies in the Search for Life on Mars" Koike *et al.*, (1995).

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