THE EFFECT OF MUTANT REPLICATION INITIATION PROTEINS AND ANTISENSE RNA TO THE GENE ON MAIZE STREAK VIRUS REPLICATION

By

TICHAONA MANGWENDE

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Signed by officials

ofessor J A Thomson
Department of Microbiology
University of Cape Town

Signed by officials

Professor E Rybicki
Department of Microbiology
University of Cape Town

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LIST OF ABBREVIATIONS

$[\alpha]$-^{32}P-dCTP	radioactive deoxycytidine triphosphate
(+)	positive
µg	microgram
ρg	picogram
µl	microlitre
µm	micrometre
A	adenine
ACMV	African Cassava Mosaic Virus
ATP	adenosine triphosphate
BMS	Black Mexican Sweetcorn
bp	base pairs
C	cytosine
Cdk	Cyclin-dependent kinase
cm	centimetres
Cpmp	Coat protein-mediated protection
dCTP	deoxycytidine triphosphate
DNA	deoxyribonucleic acid
ds	double stranded
G	guanine
hr	hours
kb	kilobase pair (s)
Log	logarithm
mg	milligram
min	minutes
mM	millimolar
mRNA	messenger ribonucleic acid
MSV-Kom	maize streak virus-isolate Komatipoort, E. Transvaal RSA
ng	nanogram
nm	nanometres
NTP	nucleotide triphosphate
OBPs	origin binding proteins
°C	degrees Celsius
PCNA	Poliferating Cell Nuclear Antigen
PCR	polymerase chain reaction
RCR	Rolling Circle Replication
SDS	sodium dodecyl sulphate

sec	seconds
ss	single stranded
SSC	salt-sodium citrate buffer
SSPE	sodium phosphate buffer
T	thymine
Tris	tris (hydroxymethyl) aminomethane
U	units
UCT	University of Cape Town
wt	wild type
IITA	International Institute of Tropical Agriculture

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LITERATURE REVIEW

1.1 GENERAL INTRODUCTION

Maize (*Zea mays* L) is a major crop plant belonging to the family *Poaceae*, which includes other important cereals such as wheat, rice, barley and oats (Kellogg, 1998). Other grass crops are rye, common millet, finger millet, teff, sugarcane and sorghum. Many of these are important food crops in most parts of the world, including Africa. The sustainable production of sufficient and affordable food for a rapidly increasing population is one of Africa's greatest challenges. Maize yields across the continent are generally low, averaging only one ton/ha in most countries. Although low input and subsistence farming are major yield constraints, diseases and insect pests aggravate the situation in two ways: direct yield loss due to crop failure and indirect yield loss through farmers reducing areas under the maize crop to avoid loss.

Maize is susceptible to a wide variety of diseases, and especially those caused by fungi and viruses. Of the viral diseases, maize streak virus (MSV) disease has attracted considerable attention. Yield losses of up to 100% have been recorded due to MSV epidemics. The maize streak disease was first identified by Fuller (1901) in South Africa. In Africa the disease correlates with the distribution of MSV's leafhopper vector, *Cicadulina* spp. (Fajemisin and Shoyinka, 1976; Rossel and Thottappilly, 1985). MSV outbreaks are erratic because they are often associated with droughts and irregular early rains (Rossel and Thottappilly, 1985).

1.1.1 Maize Streak Virus and Maize Streak Disease

The virus is the causative agent of maize streak disease. MSV particles consist of a single coat protein subunit, about 30 kDa, and have a characteristic twinned or geminate morphology of ca. 20 x 30 nm. They contain a single molecule of circular ssDNA, about 2.7-kb in size. The reason the virus particles have such a morphology is not yet known (Bisaro, 1996). While MSV particles have a sedimentation coefficient of approximately 76S, single particles have a coefficient of 54S (Bock *et al.*, 1974).

Maize streak disease is characterised by intermittent to almost continuous, narrow, chlorotic streaks, running from the leaf base to the tip, centred on secondary and tertiary leaf veins (Fajemisin and Shoyinka, 1975; see Figure 1.1). In cases excluding severe necrosis, the distribution of streaks is uniform over the leaf surface. Necrosis is a result of the complete fusion of streaks marked by irregular green lines or islands centered between veins. The genotype of a host is a determinant of symptom manifestation (Martin *et al.*, 2000).

In highly susceptible varieties distinct, intermittent or continuous, white chlorotic spots and stripes develop within 4-5 days after infection. Highly resistant genotypes are characterised by the absence of distinct streaking. Maize plants are vulnerable to MSV from emergence to tasseling, although the effect and extent of damage is variable. Plants infected within 3 weeks of emergence become stunted and may die prematurely or produce small and poorly filled ears. Plants outgrow the deleterious effects of MSV if infection occurs 6-8 weeks after emergence. Although streak symptoms in maize are quite typical they are often confused with streaks caused by *Maize stripe virus* and *Maize mosaic virus* (MMV).

Figure 1.1. Maize plant showing the streaking symptoms characteristic of the maize streak disease. The streaks start from the base of the leaf and in some areas they appear as patches of severe chlorosis (Alejandro, 1987).

MSV is distributed throughout Africa and the adjacent Indian Ocean islands. Similar virus diseases with an unknown relationship with MSV have been observed on pearl millet (*Pennisetum americanum*; P. *typhoides*), wheat (*Triticum aestivum*) and other cultivated species in India (Seth *et al.*, 1971; 1972; Seth and Singh, 1975; Raychandari *et al.*, 1976). MSV is an ecologically ubiquitous disease of maize occurring in forests and savannah grasslands, from sea level up to elevations of 2000 m (Kenyan Highlands) (Kytere, 1995).

1.1.2 The Epidemiology of Maize Streak Virus Disease

Because MSV is obligately transmitted by *Cicadulina* (see Figure 1.2) leafhoppers, MSV incidence is associated with leafhopper population dynamics. Leafhoppers are delicate insects with special feeding habits and mating behaviour which are significant factors in the incidence and distribution of maize streak disease. The population dynamics of leafhoppers is greatly influenced by rainfall, temperature and the availability of alternative hosts. Few studies have addressed the general patterns of MSV outbreaks although isolated cases have been recorded. Epidemics were reported in Nigeria in 1966, 1971, 1973 and 1976 (Esenam, 1967; Fajemisin and Shoyinka, 1976; Kim *et al.*, 1981). Severe epidemics were also reported by IITA in 1986 from the Democratic Republic of Congo (formerly Zaire), Zambia, Angola and Mozambique. IITA has also compiled reports of MSV epidemics from most African countries such as Ethiopia, Ghana, Zimbabwe, Tanzania, Togo, Benin, Burkina Faso, Sao Tome, Kenya, Sudan and Niger.

Figure 1.2. The species of *Cicadulina* leafhopper a vector of MSV distinguished by its characteristic round head with two distinct spots on the anterior margin (Alejandro, 1987).

The distribution of *Cicadulina* species varies with seasons and correlates with the incidence of maize streak disease in the field. The density of leafhoppers is determined by the number of grass hosts suitable for the oviposition and development of nymphs. Both of these factors are directly affected by rainfall. In general *Cicadulina* numbers increase with rainfall in both West and Southern Africa (Rose, 1978) even in years when the rainfall is irregular. The establishment of large and stable leafhopper populations with the early rains before the onset of the season enables the efficient transmission of MSV. However, a direct correlation between leafhopper density and the level of MSV infection has not been shown.

In maize, yield loss due to MSV infection depends on the time at which plants became infected. MSV symptoms are more pronounced when plants are infected within 3 weeks of germination. Farmers can avoid severe loss by planting to avoid migrating leafhoppers landing on young plants (Rose, 1978). This is difficult to achieve, however, as it requires a high degree of co-ordination within the farming community. Lack of snychrony in this regard could result in the creation of alternative sources of infection and support of the viral infection cycle.

The vector can be controlled by the use of insecticides. For example Gaucho 70 WS (Imidacloprid) is used in the irrigated parts of South Africa and was used on late planted maize in Zimbabwe between 1988 and 1991 (Deall *et al.*, 1993). However, the use of chemical control is increasingly unattractive because of potential adverse effects on the environment. Moreover, for the resource poor farmer the cost of chemicals is prohibitive.

The development and use of MSV resistant maize cultivars is probably the most attractive way of combating MSV epidemics. This approach entails the breeding of cultivars by conventional means. In the Reunion Islands it was found there were maize cultivars resistant to MSV, and resistance has been fixed in lines which are easily crossed with other maize lines (Soto and Baddenhagen, 1982). There is however doubt about the resilience of this resistance as it has recently been shown that MSV genotypes capable of breaking this resistance exist amongst the quasi-species of MSV isolates found in Reunion (Isnard *et al.*, 1999).

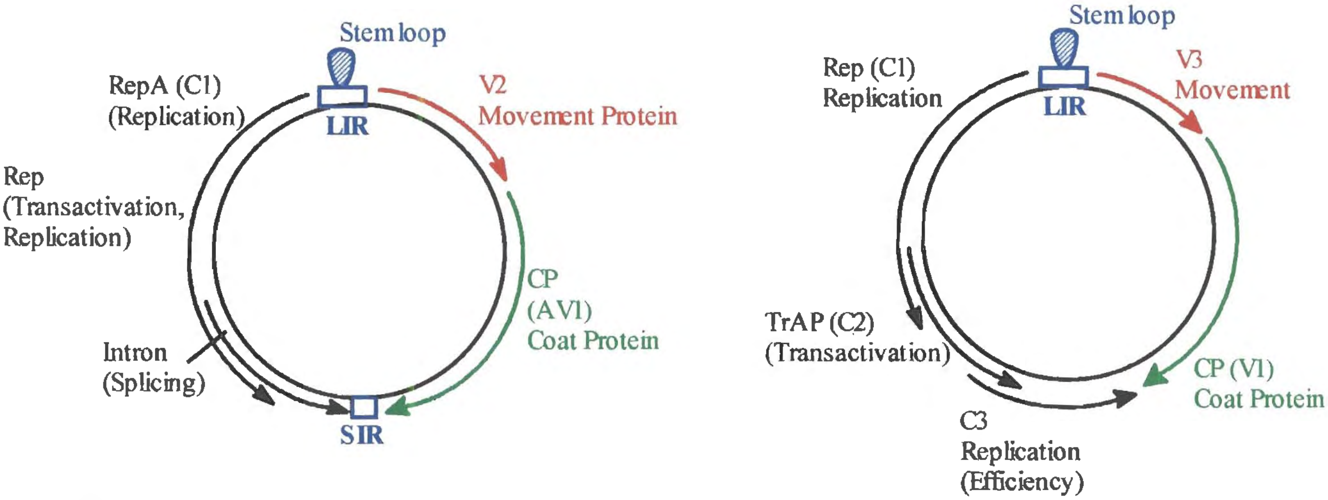
MSV resistance genes have been mapped on maize chromosomes 1 and 10 using RFLP analysis (Kytere *et al.*, 1995; Welz *et al.*, 1998). However, mapping of these genes does not make them readily usable. A high resolution genetic map including resistance genes for most of the known diseases afflicting maize is still in the developmental stages (McMullen and Simcox, 1995). As more information on the molecular biology of MSV becomes available it is hoped that a blend of molecular biology and breeding will be capable of producing durable resistance against MSV.

1.2 GEMINIVIRUSES

1.2.1 Geminivirus Genera

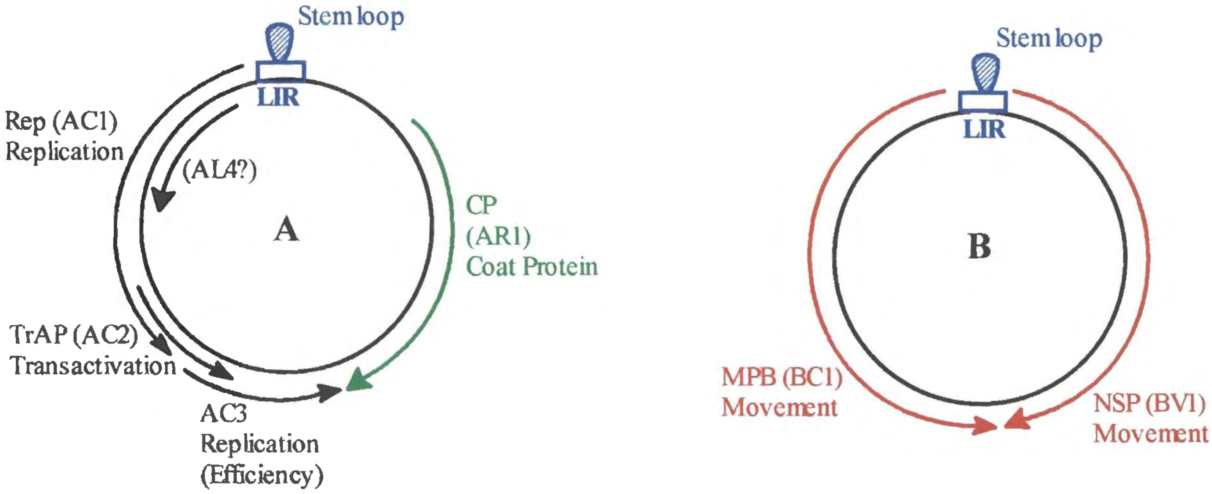
Geminiviruses are a unique group of plant viruses. Because of their circular ssDNA genomes, they replicate in the nuclei of the infected host cells, unlike positive sense RNA viruses which replicate in the cytoplasm. As of 1999 geminiviruses were classified into four genera (Figure 1.3): these were *Mastrevirus* (formerly Subgroup I), *Curtovirus* (formerly Subgroup II), *Begomovirus* (formerly Subgroup III) and the new genus Topocuvirus, according to their molecular biology, vector and host (Pringle *et al.*, 1999; Rybicki *et al.*, 2000). Mastreviruses are transmitted by leafhoppers, and infect monocotyledonous plants with the exception of *Tobacco yellow dwarf virus* (ToYDV; Morris *et al.*, 1992) and *Bean yellow dwarf virus* (BeYDV; Liu *et al.*, 1997), and have a single genome component. The genus *Mastrevirus* comprises MSV, *Wheat dwarf virus* (WDV), *Digitaria streak virus* (DSV), *Sugarcane streak virus* (SSV), *Miscanthus streak virus* (MiSV), *Panicum streak virus* (PanSV) and *Chloris striate mosaic virus* (CSMV) and several other less well characterised viruses. Curtoviruses have a monopartite genome and are transmitted by leafhoppers but infect dicotyledonous plants. Begomoviruses may have ~~monopartite or bipartite genomes, are transmitted by the~~ whitefly species, *Bemisia tabaci*, and infect dicotyledonous plants. MSV and WDV are among the best-characterised members of the mastreviruses. The curtovirus *Beet curly top virus* (BCTV) and the begomoviruses *Tomato yellow leaf curl virus*-Israel (TYLCV-

Is), *Tomato golden mosaic virus* (TGMV), *African cassava mosaic virus* (ACMV) and *Squash leaf curl virus* (SqLCV) are well characterised examples of the genera.



Mastreviruses

Curtoviruses



Begomoviruses

Figure 1.3. An illustration of the genome organizations of the three different genera of geminiviruses. The mastreviruses and curtoviruses have single genome components and the begomoviruses can have either single or double components. The topocuviruses are not included in this illustration as it is not an accepted genus (7th Report ICTV). The various ORFs are as described elsewhere in thesis.

1.2.2 Geminivirus Replication

Mastrevirus genomes consist of four open reading frames (ORFs) (Mullineaux *et al.*, 1984; MacDowell *et al.*, 1985; Donson *et al.*, 1987; Andersen *et al.*, 1988; Chatani *et al.*, 1991; Briddon *et al.*, 1992; Morris *et al.*, 1992; Hughes *et al.*, 1993; Liu *et al.*, 1997; Rybicki *et al.*, 2000). The four ORFs are in the complementary (C1 and C2) and the virion (V1 and V2) sense ORF and all are required by the viruses for replication and transmission. There is an intron in the overlap between the C1 and C2 ORFs. The Rep protein is the product of splicing of the C1:C2 transcript, whereas RepA is the product of the unspliced transcript and derives only from C1 (Wright *et al.*, 1997). The V1 and V2 ORFs are the viral movement protein (MP) and coat protein (CP) genes respectively: both proteins are essential for viral ssDNA encapsidation and systemic spread (Briddon *et al.*, 1989; Boulton *et al.*, 1989, 1993; Lazarowitz *et al.*, 1989). For the purposes of this thesis attention will be focused on the Rep-encoding ORFs.

Geminivirus replication takes place in two stages. Firstly the single-stranded (ss) DNA is converted into double-stranded (ds) DNA. The dsDNA replicative form (RF) is then used as a substrate for the viral rolling circle replication cycle (RCR) (Saunders *et al.*, 1991; Stenger *et al.*, 1991). The ssDNA product of the RCR is encapsidated by the CP to produce viral particles or is primed for second strand synthesis to produce RF DNA. The replication of geminiviruses has attracted a lot of interest because of the dependence of these viruses on the host replication machinery. The viral replication within a host nucleus is believed to be an ideal model for studying the molecular biology of plants and plant-virus interactions. Understanding the molecular biology of plant development and plant-virus interactions is vital for producing physiologically normal, fertile, disease resistant transgenic plants.

Initiation of RCR for any replicon requires a *cis*-acting DNA sequence (replicator) and a *trans*-acting protein factor (initiator). The initiator protein binds to a specific sequence of the replicator to form a specialised nucleoprotein. Geminivirus genomes contain a long intergenic region (LIR) from which the complementary and virion sense genes diverge. Most of the *cis*-acting sequences are contained in the LIR: it contains the Rep binding domain, GC-rich inverted repeats capable of forming a stem-loop or cruciform structure, and divergent RNA polymerase II type promoters (the TATA boxes) which are essential for viral gene transcription. An invariant AT rich, nonanucleotide sequence, 5'-TAATATTAC-3', in the stem-loop is found in all geminiviruses (Arguello-Astroga *et al.*, 1994; Orozco *et al.*, 1996; Figure 1.3). Rep introduces a nick in the (+) strand of the nonanucleotide between the last T and A at the 3' end in a topoisomerase type I-like manner (Bisaro, 1996). The Rep protein forms a phosphotyrosyl bond with the 5' end of the nicked + strand and remains covalently attached until the completion of one cycle. The Rep protein then terminates and ligates the newly synthesised DNA. It is not clear whether the Rep protein is reused for another round of replication or is inactivated as is the case in bacterial plasmids that utilise the RCR mechanism (Rasooly et al., 1994).

1.2.3 Geminiviruses Replication and the Host Cell Cycle

The replication of geminiviruses requires the extensive use of cellular proteins (for reviews see Lazarowitz, 1992; Bisaro, 1996; Palmer and Rybicki, 1998). Virus RF

DNAs are associated with host cell histones to form a minichromosome-like assembly during viral infection (Pilatz and Jeske, 1992; Bisaro, 1996). Acetylation and deacetylation of histones is generally associated with chromatin assembly and activity (Eden *et al.*, 1998).

Rep is not classified as a member of the DNA-binding family of proteins although it has characteristics which make it a possible member. DNA-binding proteins are mostly multifunctional, involved in several activities including replication of the genome, transcription of active genes and repairing of damaged DNA (Paubo and Sauer, 1992). One of the most important members of the DNA-binding protein group is the family of transcription factors that regulate co-ordinated gene expression. Regulation is tightly controlled and takes place during cell development, differentiation and growth. Transcription factors bind to a specific site or set of sites on promoters of genes linked to a specific cell developmental stage, resulting in activation or repression of genes. In multicellular organisms, such as plants, commitment to continued cell division or organ formation is highly regulated by transcription factors in association with other factors. Well-known families of DNA-binding proteins include those containing the helix-turn-helix, zinc finger, leucine zipper, helix-loop-helix motifs, homeodomains and steroid receptors. There are some very interesting viral proteins that bind DNA which are not covered by this seemingly general classification of DNA-binding proteins, namely the SV40 large T antigen, human p53 tumour suppressor, adenovirus 12 E1A (Ad12 E1A), human papillomavirus type 16 E7 (HPV E7) and Rep proteins of geminiviruses (Wasylyk *et al.*, 1983).

Plant and human DNA viruses' Rep and oncoproteins have similar conserved motifs but they differ in their complexity. Human DNA tumour virus oncoproteins are more complex in structure and mode of function compared to the Reps of geminiviruses and nanoviruses. Typical examples are the SV40 large T antigen and Ad12 E1A protein, which are analogous to the Rep of geminiviruses, although derived from dsDNA viruses. Although these proteins have considerable differences in complexity they are functionally conserved. Ad12 E1A is similar to the geminiviruses Rep in many respects. It is multifunctional and is an "immediate early protein" which transcriptionally activates late genes involved in the adenovirus replication cycle. The activation of genes is through interaction with transcription factors and other host cellular factors such as the human and plant retinoblastoma (Rb) protein family members (Xie *et al.*, 1996).

1.2.4 The Retinoblastoma Protein (Rb)

Plant development is a process of continuing growth and organ initiation. In animals, organogenesis is mostly completed during embryo formation. These differences mark the fundamental principles underlying the control of animal and plant development. In plants cell division occurs mostly in specialised regions known as meristems and the cambium. Cell division is highly regulated in response to developmental and environmental cues. The detailed mechanisms regulating the plant cell cycle and developmental progression have not yet been elucidated.

The Rb protein is a nuclear phosphoprotein, better characterised in animals than in plants, and not found in yeast. Both animal and plant Rb proteins have a conserved cysteine residue (amino acid 706 in human Rb). The cysteine residue probably mediates the interaction between the Rb and proteins containing the **LxCxE** or Rb-binding motif. In animals, Rb is a vital tumour suppressor protein which functions as a positive and negative regulator of cell cycle progression. The Rb exists as an active unphosphorylated or hypophosphorylated protein in the early G_1 phase and an inactive hyperphosphorylated protein in late G_1, past the restriction (R) or checkpoint of the cell cycle (Ezhevsky *et al.*, 1997). The active Rb protein exerts its regulatory effect by binding to a family of transcription factors the best studied of which is E2F (1, 2, 3, 4 and 5). It has recently been shown that the E2F transcription factors contain a conserved as yet unidentified Rb-binding motif, distinct from the well characterised **LxCxE** (for review see Dyson, 1998).

E2F is a mammalian transcription factor originally identified as one that binds specifically to a sequence element in the adenovirus E2 promoter (Yee *et al.*, 1987). E2F binds to promoters on a number of genes that are essential for cellular proliferation and DNA synthesis (DeGregori *et al.*, 1995). Factors E2F1, 2 and 3 interact strongly with the Rb protein whereas factors E2F4 and 5 interact weakly. The E2F transcription factors are vital regulators of the G_1 to S-phase transition. At elevated levels E2F drives cells from G_0 or G_1 to S-phase (Johnson *et al.*, 1993; Lukas *et al.*, 1996; for review, see Adams and Kaelin, 1996). The Rb protein inactivates E2F by binding directly to the E2F activation domain. The E2F is composed of heterodimers between one of the five E2F proteins and one of the three DP proteins (Lam and Lathangue, 1994). The Rb targets proteins containing the E2F1-, E2F2- and E2F3- heterodimers, while the other Rb family members p107, and p130, target E2F4 and E2F5-containing heterodimers (Slansky and Farnham, 1996). The interaction is mediated by the homobromodomain complex, hbrm/BRG-1, human homologues of the yeast SWI2/SNF2 proteins (Dunaief *et al.*, 1994;

Chiba *et al.*, 1994). The binding and inactivation abrogates E2F function and timeously drives cells into the S-phase. Rb proteins also bind to other cellular proteins including D cyclins, acetylases, deacetylases, RbAp48 and RbAp46. RbAp48 and RbAp46 are human nuclear proteins, which are respectively 90% and 30% identical to the yeast Msi1 protein (Multicopy suppressor of ira1). Msi1-like proteins have been implicated in the regulation of histone acetylation and deacetylation which is central to chromatin formation. Human RbAp48 is a subunit of both a histone deacetylase and a chromatin assembly factor that is involved in assembling the nucleosomes during DNA replication.

Recently cDNA clones encoding Rb-like proteins have been made from maize RNA (Grafi *et al.*, 1996; Xie *et al.*, 1996; Ach *et al.*, 1997). Prior to the work by Huntley *et al.* (1998), no evidence on the possible role of plant Rb homologues in vegetative growth and development of plants had been presented. Huntley and co-workers demonstrated the conservation of function between mammalian and plant factors involved in G_1/S-phase transition control. More importantly, ZmRb-1 expression (Xie *et al.*, 1996) has a strong correlation with cell differentiation during maize leaf development and endoreduplication of the endosperm (Huntley *et al.*, 1998; Grafi *et al.*, 1996). The existence of plant Rb homologues such as ZmRb-1, which are capable of binding and inhibiting mammalian E2F, points to the conservation of the G_1 regulatory mechanism in both plants and animals.

Plants have homologues of the mammalian D cyclins. Originally known as δ-cyclins in *Arabidopsis* (Soni *et al.*, 1995) they have now been renamed CycD (Renaudin *et al.*, 1996). Cyclins are short half-life regulatory protein subunits (Matsushime *et al.*, 1992; Sewing *et al.*, 1993; Sherr, 1996) of cyclin-dependent kinases (cdks) that are produced cyclically (hence the name cyclin). When cdks are activated by cyclins they mediate the hyperphosphorylation of Rb in both plants and animals. In *Arabidopsis*, as in the animal species so far studied, cyclins are classified as CycD (1, 2, 3, 4 and 5). CycD1 has been shown to physically bind to the maize Rb-like protein, RRB1 (Ach *et al.*, 1997). Cdks are active only when they form complexes with a cyclin. The cdk4/cyclin D1 specifically mediates the phosphorylation of serine residues in mammalian Rb (Connell-Crowley *et al.*, 1997; Kitagawa *et al.*, 1996). ZmRb1 contains sixteen consensus S/TP sites for cdk phosphorylation (Xie *et al.*, 1996). As cdks are highly conserved and found in all eukaryotes analysed to date, including several plant species, the cdk/cyclin activated complex combination is a prerequisite for Rb protein phosphorylation and is also probably conserved across a wide genetic divide. Cdc2-like genes, functionally homologous to cdks, have been analysed from *Arabidopsis* (Ferreira *et al.*, 1991; Hirayama *et al.*, 1991), alfalfa (Hirt *et al.*, 1991), pea (Feiler and Jacobs, 1990) and maize (Colasanti *et al.*, 1991). These cdks can all complement cdc2 mutants in *Saccharomyces pombe* or mutants in the cdc28 (an equivalent gene) in *Saccharomyces cerevisiae* (Martinez *et al.*, 1992). As the cdks play a critical role in cell cycle control the analysis of the cdc2-like genes in *Arabidopsis* has provided the basis for studying the mechanism of regulation of cell proliferation in relation to stem cell organisation.

Plant homologues of human RbAp48, RbAp46 and yeast Msi1 were isolated using degenerate PCR primers corresponding to two conserved regions of the three proteins. Libraries (cDNA) from young tomato fruit and *Arabidopsis* leaves were used for PCR amplification. The high sequence identity among the plant Msi1-like proteins shows that they are fairly conserved between monocots and dicots. The association of

RbAp48 with histone deacetylase (Tanton *et al.*, 1996) raises the possibility of RbAp48 serving as an adapter that targets the deacetylase to specific regions of the chromatin. Of particular interest is the interaction of RbAp48 with Rb and hypoacetylated histones, which are features commonly associated with inactive transcriptional domains (Wolffe and Pruss, 1996). This might mean that the interaction between Rb and RbAp48 supplements some of the transcriptional repression effects of Rb by targeting specific regions of the chromatin for deacetylation. From this information it appears that Rb-like proteins have as important a function in the chromatin-dependent regulation of RNA polymerase II transcriptional activity as they do in the general regulation of RNA polymerase I and II activities (White, 1997). The proteins serve as histone chaperones during chromatin formation and maturation (Roth and Allis, 1996). Nucleosome assembly and deposition are critical features of S-phase cells. The binding of RbAp48 and MSI proteins by Rb and RRB1 possibly assist in sequestering the histone chaperones during G_1. This helps in keeping aspects of the nucleosome metabolism on hold until Rb is phosphorylated at the (R) point and the cell is ready to begin DNA synthesis.

1.2.5 Interactions between the Retinoblastoma Protein and Other Plant-Associated Proteins

Plant Msi1-like genes are the third group of genes cloned from plants that encode proteins related to the mammalian Rb-binding proteins. Although neoplastic transformation is a rare event in plants the cell cycle in plant roots is reactivated by *Rhizobium* Nod factors (Yang *et al.*, 1994) and *Agrobacterium tumefaciens* infection results in formation of typical crown galls (Kado, 1991). The VirB4 protein encoded by

Agrobacterium tumefaciens and *Agrobacterium rhizogenes* contains the **LxCxE** motif. The VirB4 protein is required for tumour induction and it probably functions through the **LxCxE** (Hooykas and Beijersbergen, 1994). The domain that mediates this interaction has not yet been identified. Of notable interest are the cyclin genes cloned from *Arabidopsis* that are homologues of the animal D cyclins. The plant CycD and animal D cyclins contain a conserved **LxCxE** binding motif, usually preceeded by a glutamic or aspartic amino acid residue, at their N termini (Soni *et al.*, 1995). In human D1 cyclin this has a misspaced superimposed **LxCCxE** (Dowdy *et al.*, 1993). *Arabidopsis* CycD1 binds to the maize RRB1 and ZmRb-1 proteins presumably via the conserved Rb-binding motif (Ach *et al.*, 1997; Huntley *et al.*, 1998). The **LxCxE** motifs present in known Rb-interacting proteins are shown in Table 1.1.

Table 1.1. The conserved amino acid sequences of the Rb-binding domains of oncoproteins, plant cyclins and viral Rep proteins.

Source	Protein	Amino acid sequence
Ad12	E1A	EDMD**L**L**C**Y**E**MGFPCSDSEDE
SV40	T-Ag	**E** EN**L**F**C**S**E**EMPSSDDE
HPV-16	E7	ETTD**L**Y**C**S**E**QLNDSSEEE
Arab	CycD1	NDMD**L**F**C**G**E**DSGVFSGES
Arab	CycD2	MAEN**L**A**C**G**E**TASHGSLTT
Arab	CycD3	LLDA**L**Y**C**E**E**EKWDDECEEE
Human	cyclin D1	MEHQ**LLC**C**E**VETIRRAYP
Human	cyclin D2	ME**LLC**H**E**VPDVRRAVR
Human	cyclin D3	ME**LLC**C**E**GTRHAPRAG
BBTV	C5	VYQD**L**Y**C**D**E**VLSSSSTEEED
MDV	C4	DMDD**L**S**C**G**E**LFIPDEEDDLT
SCSV	C3	MED**L**Y**C**S**E**LGFPNYGEDD
MSV	Rep	SSPD**LLC**N**E**SINDWLQPNIF
WDV	Rep	PTES**L**I**C**H**E**TIESWKNEHL
DSV	Rep	SEPD**LLC**Q**E**TITDWLQNDL

Animal DNA virus oncoproteins such as the SV40 large T-antigen, Ad12 E1A and HPV16 E7 have been shown to bind Rb in virus transformed cells (Whyte *et al.*, 1989; DeCaprio *et al.*, 1988; Dyson *et al.*, 1989). The viral proteins SV40 large T-antigen, Ad12 E1A and HPV16 E7 ~~proteins form stable complexes with the Rb protein~~ and inactivate it. This function closely resembles the cyclin-mediated phosphorylation of Rb. The interaction is through the A and B pocket domains of Rb via the **LxCxE** binding motif. In viral oncoproteins alteration of the conserved **L**, **C** or **E** amino acid residues results in a dramatic decrease in binding of the viral protein to Rb (Moran *et al.*, 1986; Lillie *et al.*, 1987; Cherington *et al.*, 1988; DeCaprio *et al.*, 1988; Moran, 1988; Smith and Ziff, 1988). An exception is the large T-antigen of the papovavirus, avian budgerigar fledging disease virus that contains a C→A change within a functional Rb binding domain (Pipas, 1992).

In geminiviruses, mastreviruses encode an initiator protein RepA (C1 ORF) absolutely required for replication. The replication of mastreviruses, except SSV, which does not have an obvious Rb-binding motif, apparently depends on an intact **LxCxE** of RepA (Xie *et al.*, 1995). The RepA can bind to human Rb, p107^{Rb} (Collin *et al.*, 1996), human homologue p130^{Rbr2} protein (Xie *et al.*, 1995) and to the maize ZmRb1 (Grafi *et al.*, 1996; Xie *et al.*, 1996). Binding assays using the yeast two-hybrid system demonstrated that interaction of ZmRb1 and RepA is through the **LxCxE** motif. Xie *et al.* (1995) have shown that changing the glutamic amino acid to lysine in the **LxCxE** motif of RepA completely abolishes replication of WDV. In TYDV mutations of the conserved glutamic acid reduced the binding capacity of RepA to the Rb protein by 95% but the mutant was infectious (Liu *et al.*, 1999). The residual interaction between Rb and the mutated Rep protein is not productive. Despite the fact that the Rep protein of the begomovirus TGMV does not have the **LxCxE** motif, it has been shown to interact with the maize RRB1 (Ach *et al.*, 1997). In addition the TGMV Rep, upon viral infection, drives cells into the S-phase and induces the synthesis of the proliferation cell nuclear antigen (PCNA) in terminally differentiated cells (Nagar *et al.*, 1995). The PCNA is an accessory factor for DNA polymerase δ processivity and is a marker for cellular DNA synthesis. This probably means the regulation of TGMV replication and transcription does not require the function of the **LxCxE** motif. Interaction between the Rep of begomoviruses and Rb is probably through an as yet unidentified domain.

While missing the **LxCxE** motif, curto- and begomoviruses have additional genes, TrAP and REn, encoded by C2 and C3 or AC2 and AC3 ORFs respectively (Elmer *et al.*, 1988; Sunter *et al.*, 1990; Rybicki *et al.*, 2000). TrAP is a *trans*- acting activator of the virion-sense promoters (Sunter and Bisaro, 1991) and REn is a replication enhancer (in curto- and begomoviruses; Figure 1.3). What has not yet been demonstrated for these viruses is the form of molecular interactions, which link their replication cycles with that of their host in order to establish an environment permissive to viral replication. In the curtoviruses the link is probably via the C4 protein interacting with host factors as demonstrated for the BCTV (Latham *et al.*, 1997).

MSV Rep, in addition to having several other functional motifs, has a myb-like binding motif in the Rep C-terminus (Hofer *et al.*, 1992). Mybs are transcription factors, which contain a domain structurally and functionally related to the DNA binding domain of the retroviral oncogene *v-myb* and its cellular homologue *c-myb* (Kranz *et al.*, 1998). Myb proteins of animals, yeast and plants consist of well-conserved domains of 50 to 53 imperfect repeats of amino acids (Rosinski and Atchley, 1998). The first plant *myb* gene to be identified was *C1* from *Zea mays* (Paz-Ares *et al.*, 1987). The C1 transcription factor regulates the synthesis of anthocyanins in the aleurone layer of *Z. mays* kernels. It is possible that the myb proteins interact with Rep. The possibility of this interaction has important bearings on the development of transgenic plants expressing a wild type Rep transgene. Mybs are transcription factors in plants known to control the expression of up to a hundred genes even in *Arabidopsis* with its small genome. The genes controlled by the myb transcription factors are those mainly involved in embryogenesis, activation of dormancy, trychome formation, epidermal shape, seed dessication tolerance and seed maturation (Kirik *et al.*, 1998). While embryogenesis is a very vital process in normal plant development, seed development has a great impact on the fertility and yield of an otherwise physiologically normal plant. If MSV Rep interacts with myb transcription factors it is reasonable to speculate that expression of wild type Rep will result in a viral infection phenotype. This has been observed in tobacco plants expressing the TYLCV wild type Rep (Brunetti *et al.*, 1997). It is likely that the production of transgenic plants with varied aberrant phenotypes could be a result of a variety of interactions between Rep and host factors.

1.2.6 The Plant Viruses: Infection Cycles

Viruses consist of a set of one or more nucleic acid template molecules, normally encased in a protective coat, or coats of protein (CP (s)) or lipoprotein and are capable of replicating in suitable host cells. The genome of a virus carries the genetic information necessary for its replication, movement and encapsidation. The genetic material is composed of RNA in most plant viruses, or DNA in the case of caulimo-, badna-, nano-, or geminiviruses (see Hull, 1999; Sano *et al.*, 1998; Hanley-Bowdoin *et al.*, 1999). The majority of plant RNA viruses have single stranded (ss) genomes except for the dsRNA cryptoviruses and viruses of the family *Reoviridae*. Caulimoviruses, some geminiviruses and a number of RNA plant virus groups have genomes composed of a single molecule of nucleic acid, and these are referred to as monopartite. Some RNA and DNA viruses have two or more nucleic acid species that are encapsidated, and these are referred to as bi-, tri- or multipartite (Murphy, 1996; Hanley-Bowdoin *et al.*, 1999; Stanley *et al.*, 1999; Sano *et al.*, 1998).

Genomes with more than one molecular species of RNA or DNA usually require the presence of all their major genomic components for complete infectivity. The *Tomato spotted wilt virus* (TSWV) and reoviruses have multipartite segmented genomes but are encapsidated within the same protein shell (De Haan *et al.*, 1989). The division of genetic information into two or more parts is a feature found exclusively in certain ssRNA viruses and gemini- and nanoviruses. The arrangement of divided genomes in multipartite viruses is advantageous in that it allows the virus particle to remain small and easily protected by the CP. The division of RNA genomes into a number of short RNA strands reduces lethal errors that may occur during replication: in short strands of RNA this error will affect only one strand. Plant viruses, DNA or RNA have similar infection cycles despite differences in their genome organisation, vectors, hosts and replication strategies (see Figures 1.4 and 1.5).

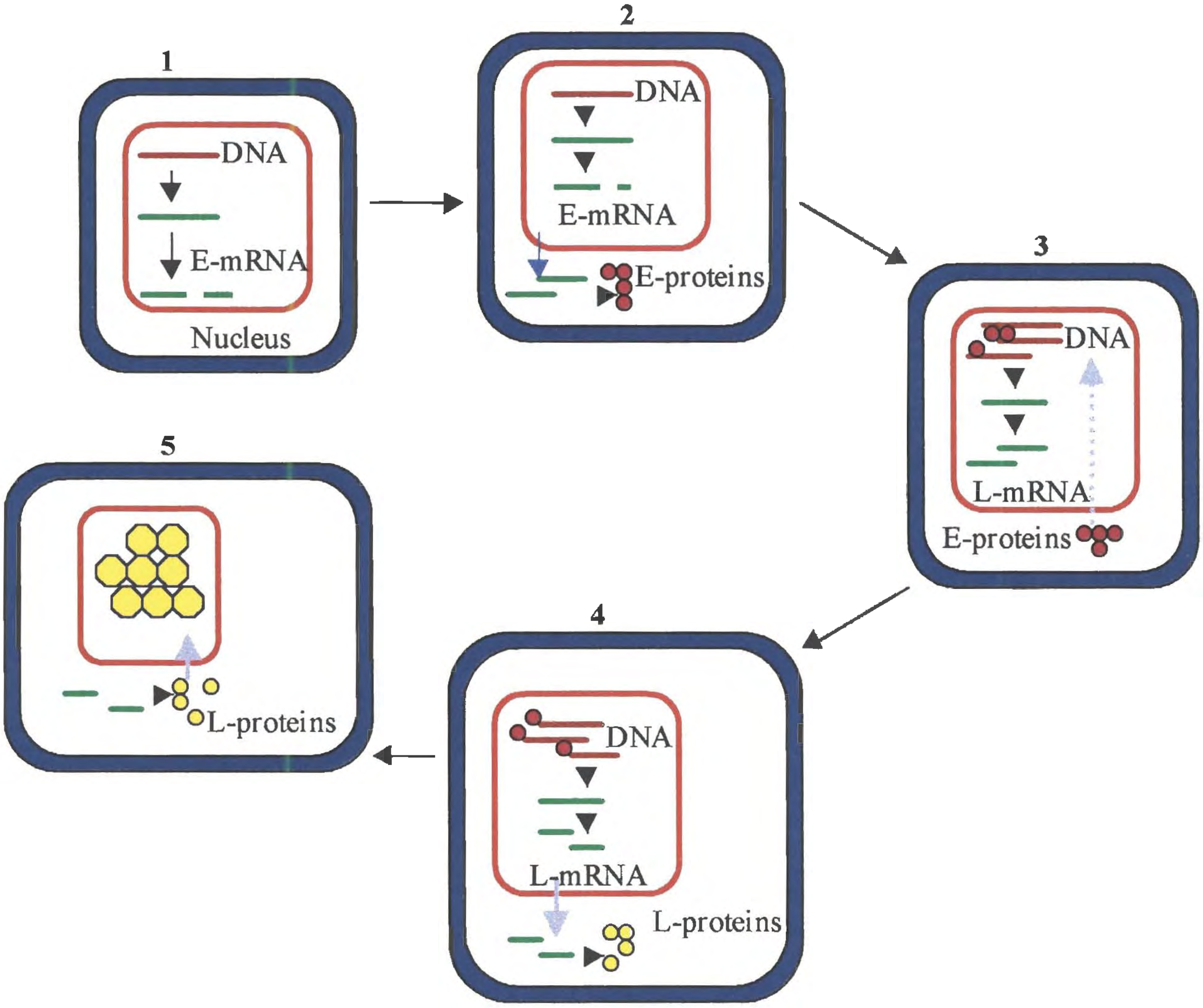


Figure 1.4. A simplified infection-cycle of a DNA virus for example a geminivirus. The stages marked are; 1, Uncoating or disassembly of the virus particle, transcription and processing of early mRNA (E-mRNA) in the nucleus; 2, transport of "E-mRNA" to the cytoplasm and translation into early proteins (E-proteins); 3, transport of E-proteins to the nucleus, replication of viral DNA, synthesis and processing of "late-mRNA" (L-mRNA); 4, transport of L-mRNAs to the cytoplasm and translation into structural proteins; 5, transport of L-proteins into the nucleus and assembly of virus particles.

The stages of the life cycle tend to differ between the RNA and DNA viruses, with the DNA virus having multiple stages of shuttling (DNA, RNA and proteins) in and out of the nucleus. The infection cycle of RNA viruses in Figure 1.5 has three main stages compared to that of the DNA virus (Figure 1.4) that has up to five stages.

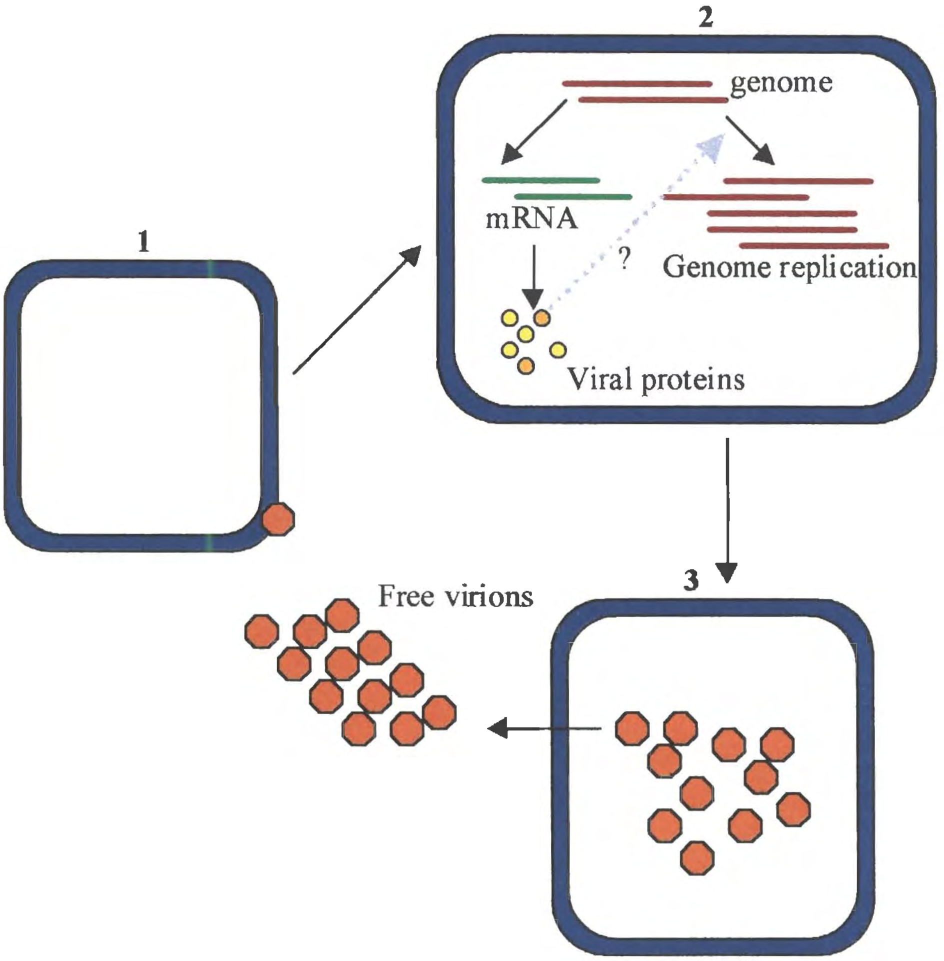


Figure 1.5. A simplified infection cycle of a plant RNA virus. The stages numbered are; 1, transmission of the virus through feeding by aphids for example; 2, virus disassembly, synthesis and replication in the cell cytoplasm; 3, virus particles are assembled and the transmission starts all over again.

For a virus to survive, it needs a permissive plant host in which it can multiply and be transmitted. In order to maintain itself the virus must utilise an effective means of transmission from cell to cell within an infected plant and from one host to another. The need for the virus to survive and multiply entails complex interactions between physical and biological factors. The infection cycle of a plant virus can be divided into two main sections, namely transmission into host plant and multiplication within the host plant (see Figures 1.4 and 1.5). The transmission of most plant viruses by a vector takes place during feeding. The vectors include aphids, leafhoppers, planthoppers, treehoppers, whiteflies and mealy bugs, which feed by sucking on green plants (Tonkyn and Whitcomb, 1987). The virus transmission may be divided into three basic types, non-persistent, semi-persistent and persistent. Non-persistent transmission is characterised by short acquisition feeding time and rapid loss of the vector's ability to transmit. Aphids transmit viruses in a non-persistent manner and the viruses are normally carried on or near the mouthparts (Harris, 1977, 1979; Pirone and Harris, 1977; Murant *et al.*, 1976).

1.3 PATHOGEN-DERIVED RESISTANCE IN PLANTS

Pathogen-derived resistance (PDR) is the transformation of susceptible plants with portions of the viral genome to produce plants that are resistant to the virus from which the sequences are derived (for review see Lomonossoff, 1995). The PDR concept was first proposed by Hamilton in 1980 and further developed by Sanford and Johnson in 1985. The idea of producing genetically engineered virus resistant plants using PDR evolved from empirical observations on the use of mild or attenuated strains of viruses to protect field crops (Wilson, 1993). Field crops such as tomato, apple, citrus, papaya and tobacco were protected against closely related pathogenic viruses using such strains for cross-protection (Beachy *et al.*, 1990; Gadani *et al.*, 1990; Hull and Davies, 1992).

PDR can either be RNA or protein-mediated. Protein-mediated resistance is the expression of a functional or dysfunctional viral gene product (structural or non-structural) that does not support disassembly, transcription, translation, replication or spread of the virus. Most examples of protein-mediated protection have focused on the use of the CP although there are other examples of the MP and replicase protein-mediated resistance. A dysfunctional protein interferes with the viral infection cycle in a "dominant negative" manner, for example the movement protein of TMV (Malyshenko *et al.*, 1993). In RNA-mediated resistance, mostly in RNA viruses, untranslatable RNA sequences of the target gene that could be CP or the replicase protein sometimes result in highly resistant phenotypes. This has been shown against *Tomato spotted wilt virus* (TSWV) and *Tobacco etch virus* (TEV) (Pang *et al.*, 1992; Lindbo and Doughtery, 1992).

The PDR concept was extended to include the dominant negative mutation of viral nonstructural genes, for example the Reps of geminiviruses (Noris *et al.*, 1996; Hong and Stanley, 1996; Brunnetti *et al.*, 1997; Sangare *et al.*, 1999). A non-functional protein for example a truncated Rep acts as a "negative-dominant factor" by facilitating a non-productive interaction with the origin of DNA replication out-competing wild type Rep from the incoming virus. In-depth reviews of PDR against a variety of plant viruses have been published (for reviews see Wilson *et al.*, 1993; Scholthof *et al.*, 1993; Fitchen and Beachy, 1993; Lomonossoff, 1995). RNA mediated resistance will be briefly considered in this Chapter and antisense RNA will be discussed elsewhere in this thesis. The use of "dominant negative" mutants as a resistance strategy against geminiviruses will not be considered here but in other Chapters of this thesis.

1.3.1 RNA-Mediated Resistance

There are four RNA based strategies namely; sense RNA, antisense RNA, defective-interfering (DI) RNA or satellite RNA. Sense RNA-mediated or homology-dependent virus resistance is distinct from CPMP, or resistance via dominant negative mutants in that the resistance is conferred by expressing transgenes that code for untranslatable RNA homologous to viral RNA sequences (Baulcombe, 1996; 1999; Prins and Goldbach, 1996). Antisense RNA, DI RNA (Kollar *et al.*, 1993) and satellite RNA (Harrison *et al.*, 1987) conferred a minimal level of resistance and will not be discussed in this part of the thesis. The sense RNA-mediated resistance suprisingly conferred complete resistance (van der Vlugt *et al.*, 1992; Lindbo and Dougherty, 1992; Smith *et al.*, 1994; Whitty *et al.*, 1994). Sense RNA leads to post-transcriptional silencing characterised by a dramatic sense or co-suppression of virus replication (van der Vlugt *et al.*, 1992; Smith *et al.*, 1994; Baulcombe, 1996; van Kammen, 1997; van den Boogaart *et al.*, 1998). The untranslatable RNA is generated by deletion of the AUG initiation codon, changing the initiation codon into a sense codon, or introduction of termination codons downstream of the initiation codon (Lindbo and Dougherty, 1992). This sense RNA strategy triggers a "cellular system" which specifically eliminates particular RNA sequences (Smith *et al.*, 1994; Dougherty and Parks, 1995; Lindbo *et al.*, 1993).

The resistance mediated by post-transcriptional silencing is referred to as "immunity" and is known to be active in virus-free developing leaves of plants previously challenged with virus (Dougherty and Parks, 1995). This means that the nature of silencing is not constitutive but is developmentally regulated. The phenotypes "immunity" and "recovery" are different aspects of post-transcriptional silencing (Tanzer *et al.*, 1997). The mechanism of post-transcriptional gene silencing and RNA-mediated viruses resistance is explained by two models "quantitative or threshold" and "qualitative or aberrant RNA" (Lindbo *et al.*, 1993; English *et al.*, 1996). In the "threshold" model the accumulation of transgene RNA above a threshold level triggers a cytoplasmic program that degrades all RNAs containing sequences homologous to those of the transgene. In the "aberrant" model an as yet unknown feature of the transgene provokes the production of RNAs that are in some way aberrant. This anomalous feature triggers the degradation of all related sequences (English *et al.*, 1996). The aberrant RNA is likely to be a product of ectopic pairing of homologous DNA when the insert copies contain inverted repeat copies (van Blockland *et al.*, 1994; Baulcombe and English, 1996; Sijen *et al.*, 1996; Stam *et al.*, 1997) and transgene methylation. These two models have been proposed and they incooperate new cases of post-transcriptional gene silencing (Depicker and van Montagu, 1997; Wassenenger and Pelissier, 1998). An interesting example of silencing is the interaction of the *Plum pox potyvirus* (PPV) with the NIb replicase transgene cistron to induce and maintain resistance in *Nicotiana bethamiana* plants (Guo *et al.*, 1999).

Cells of higher eukaryotes are known to express nuclear double stranded RNA (dsRNA). The dsRNA species are found in the nuclear and cytoplasmic cellular compartments of the host plant or animal cell: In the cytoplasm, dsRNAs do not naturally exist and their presence is most likely due to viral infections. The sense RNA strategy is based on induction of the host cell "surveillance system" (Waterhouse *et al.*, 1998). The nature and location of the duplex RNA determine the cell's "surveillance system". In animal cells cytoplasmic dsRNA induces cell defense response using the interferon response pathway whereas nuclear dsRNA does not. Nuclear dsRNA has an important post-transcriptional regulatory role, which controls the level of viral transcripts (Kumar and Carmichael, 1998).

Nuclear dsRNA is a good substrate for enzymes which deaminate adenosine residues to inosine within the polynucleotide structure, this results in partial or full unwinding of the duplex (Scadder and Smith, 1997). Extensive modification of RNA results in rapid degradation or retention in the nucleus. RNA with minor modifications may be transported to the cytoplasm were they produce altered proteins. The novel ribonuclease (I-RNase) is specific for inosine containing RNA and degrades transcripts with all guanosine residues replaced by inosine at an accelerated rate (Scadden and Smith, 1997). The $K_{m'}$ for I-RNase with the K_i for inhibition by normal RNA suggests a 300-fold preferential binding to I-RNA, which accounts for the specificity of degradation. RNA is modified *in vitro* by the dsRNA adenosine deaminase (dsRAD). *In vivo* dsRAD is induced by interferon and they destabilize dsRNA by converting adenosine to inosine. I-RNase in concert with dsRAD is probably part of a novel cellular antiviral defense mechanism that acts to degrade dsRNA.

1.4 THE AIMS OF THIS STUDY

The aims of this study are as follows:

(i) To introduce point mutations using abutting primer PCR site-directed mutagenesis to the modular functional domains of the MSV-Kom (Maize streak virus-isolate Komatipoort) Rep or RepA and predict the probable secondary structure using the Chou-Fasman Program.

(ii) To make replicative constructs (agroinfectious) containing the mutated MSV-Kom Rep derived sequences for analysis of the replication of MSV-Kom. This is necessary to evaluate the effect of the mutations on MSV-Kom infectivity, replication and symptom development.

(iii) To construct plant transformation vectors, containing the MSV-Kom Rep sequences for potential *trans*-dominant inhibition of MSV-Kom replication in Black Mexican sweetcorn (BMS) suspension cells and possible use in transgenic maize.

(iv) To study the inhibitory effect of antisense mRNA utilising the MSV-Kom full-length Rep sequences under promoters of different strengths.

CHAPTER TWO

CONTENTS

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CHAPTER TWO

Abutting Primer PCR Site-Directed Mutagenesis of the Conserved Functional Domains of MSV Replication Initiation Protein (Rep)

ABSTRACT

The Rep and RepA proteins of MSV consist of several distinct domains with diverse biochemical activities, among them the origin of replication, recognition and specificity, nicking-closing, oligomerization, transactivation, nuclear localization signal, Rb-binding and the NTP/ATPase-helicase. Plasmid constructs carrying single, double or triple mutations to the nicking-closing, Rb and NTP/ATPase-helicase motifs respectively, were developed using abutting primer (AbP)-PCR mutagenesis. Using the Chou-Fasman program the possible secondary structures of the wild type and mutant Rep or RepA proteins were predicted. Mutating individual amino acids $D^{99}\rightarrow A$, $Y^{100}\rightarrow F$ and $I^{101}\rightarrow F$ in MSV-Kom Rep introduced hydrophobicity within the nicking-closing domain, no detectable changes and probable extensive conformational changes within the C-terminal region respectively. The mutants $D^{99}\rightarrow AYI^{101}\rightarrow F$ and $N^{201}\rightarrow LE^{202}\rightarrow K$ produced identical putative conformational changes within the Rb-binding motif although different domains were mutated. Changing the variant amino acids $Q^{248}\rightarrow PN^{249}\rightarrow Y$ within the NTP/ATPase-helicase motif resulted in possibly extensive conformational changes. The mutated residues are four amino acids upstream of the probable transactivation motif of the Rep protein. The probable implications of the structural changes to the inhibition of MSV replication are discussed.

2.0 INTRODUCTION

Replication is a vital stage in the infection cycle of a virus or replicon. The initial step in DNA replication is the binding of an initiator protein to a discrete DNA site; namely, the origin of replication (Jacob *et al.*, 1963; Stillman, 1993). The binding is replicon or virus-specific. In the replicon pT181, the homodimeric initiator protein RepC/C induces an extrusion of the origin of replication sequence as a cruciform whose nicking site is at the tip (Jin *et al.*, 1997). Binding is followed by origin-specific unwinding and recruitment of other proteins to form a pre-initiation complex or replisome (for review see Borowiec *et al.*, 1990; Kelman and O'Donnel, 1994; Stillman, 1994). Initiator proteins, alternatively known as the origin binding proteins (OBPs), are known to nucleate the assembly of the DNA replication apparatus through specific interactions with other replication proteins. The structure of a distorted, unwound origin and the mechanism for origin unwinding have been studied in Epstein-Barr virus nuclear antigen 1 (EBNA1) and OBP from EBV a herpesvirus, E2 protein from a papillomavirus, and T-ag from SV40 (Bochkarev *et al.*, 1996; Hedge *et al.*, 1992; Bochkarev *et al.*, 1995). The interaction of T-ag with the SV-40 origin of replication has served as a model for studies of origin recognition in eukaryotic organisms (Borowiec *et al.*, 1990; Fanning and Knippers, 1992).

The origin of replication in geminiviruses is located in the large intergenic region and includes a binding site for Rep and the initiation site for (+) strand DNA synthesis located at the tip of a possible cruciform. In begomoviruses the Rep binding site mediates origin recognition and auto-repression of the complementary-sense promoter (Eagle *et al.*, 1994; Fontes *et al.*, 1994). The Rep protein of mastre-, curto-, and begomoviruses and its interaction with the predicted origin binding site are implicated in virus-specific origin recognition (Arguello-Astorga *et al.*, 1994; Choi and Stenger, 1996; Sanz-Burgos and Gutierrez, 1998). A distinct contribution of the N-terminus of the Rep protein was predicted for 17 dicot-infecting geminiviruses using the EMBL Predict program (Rost and Sander, 1993). Of particular interest is the high degree of homology between helices 1 and 2 among different geminiviruses, especially helix 2 which is conserved at 9 of the 11 positions and displays strong amphipathic character (Orozco *et al.*, 1997). The Rep protein of MSV-Kom has an α-helical region between amino acids 32 and 43. The α-helical regions are characteristic of DNA binding motifs involved in origin recognition and binding (Pabo and Sauer, 1992).

In this investigation the objective was to produce mutants that keep the origin-binding domain ("primary function") unchanged while changing other functions ("secondary") to facilitate competitive binding of the Rep protein to the origin. Site-directed mutagenesis is a useful tool for producing mutants to study the role of specific amino acid residues in the structure or function of a protein. There are well-established PCR-based methods for generating site-directed mutations (Chen and Przybyla, 1994; Hemsley *et al.*, 1989; Hughes and Andrew, 1996; Jones and Howard, 1990; Liang *et al.*, 1995).

I report the use of AbP-PCR to introduce various mutant combinations in the MSV-Kom Rep gene using the modified methods of Weiner *et al.*, (1994), and Briddon *et al.*, (1993). The probable secondary structures of the mutant MSV-Kom Rep and RepA proteins were predicted using the Chou-Fasman program (1978). The use of these mutant proteins in studying MSV-Kom replication and a possible resistance strategy are discussed.

2.1 MATERIALS AND METHODS

2.1.1 Abutting Primer PCR Site-Directed Mutagenesis

To generate mutants of MSV-Kom Rep, plasmid pKEP*rep*4 was used as a template. It contains the entire wild type MSV-Kom Rep gene cloned in the *Bam*HI-*Eco*RV site of the vector pKS. Using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) with minor modifications, I increased the mutagenesis efficiency. I used a modified version of the method of Weiner *et al.* (1994) and achieved a more than 80% increase in mutagenesis efficiency compared with published data (Fischer and Pei, 1997). The method has the advantage of using any vector with two specific primers in a single PCR reaction. In less than 48 hr, mutation-carrying colonies were obtained. Many of the laboratory strains of *E.coli* are *dam+* and plasmids prepared from them are methylated. *Dpn*I, which cleaves 5'-GA$^{m6\downarrow}$TC-3' methylated and hemimethylated substrates (New England Biolabs, 1996), does not cut newly synthesized plasmid DNA as it is not methylated. Cleavage of the parental plasmid reduced the background colonies. The omission ~~of *Dpn*I digestion reduced the mutagenetic~~ efficiency from more than 80% to less than 40%, a result similar to previous work (Fischer and Pei, 1997). The PCR procedure is shown in Figure 2.1.

Twenty ng of pKEP*rep*4 was used as template DNA for the first round of PCR with different combinations of primers (Table 2.1) to generate various single mutations. In subsequent PCR reactions to introduce double or triple mutations, plasmids carrying single or double mutations were used as templates. The PCR reactions were set up according to the supplier's instructions. The PCR cycling conditions were: denaturing of DNA at 95°C for 2 min followed by 12 cycles at 95°C for 30 sec, 55°C for 30 sec, 68°C for 12 min and completion of reactions at 72°C for 10 min. The abutting primer PCR protocol I used does not produce circular dsDNA with staggered nicks; therefore, ligation of linear PCR products was carried out using the Fast-Link ligation kit (Epicentre Technologies Inc., USA) without phenol-chloroform extraction and ethanol precipitation (Fisher and Pei, 1997). After ligation the residual parental plasmid from PCR amplification was digested with 10 U of *Dpn*I at 37°C for 2 hr. The digestion mixture was used to transform competent *E. coli* DH5α cells and putative mutation-carrying clones were analysed using PCR. Ten ng of DNA from each of the putative clones was

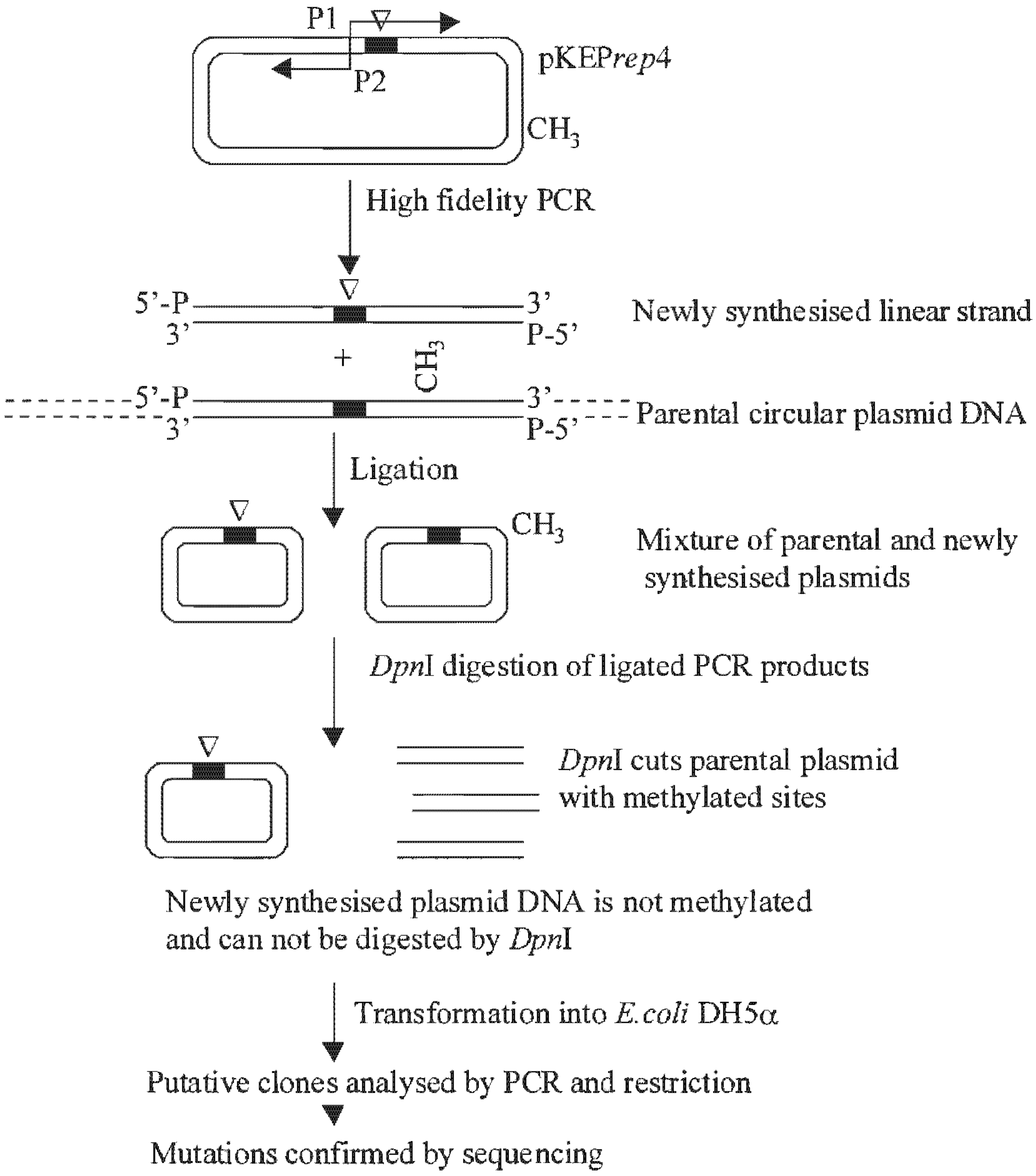


Figure 2.1. An overview of the abutting primer PCR site-directed mutagenesis strategy. It is a modified version of the staggered primer PCR-based method using *Pfu* DNA polymerase with a pair of mutagenic and non-mutagenic primers. The thermocycling conditions used are described in Materials and Methods. To generate double or triple mutations the mutagenesis procedure was repeated using a template already carrying a desired single or double mutation as a template for PCR. ∇, point mutation (see Table 2.1); P1 and P2, 5' phophorylated primers; ■, the Rep domain to be mutated.

Table 2.1. Primers used for PCR site-directed mutagenesis of the amino acid residues of the conserved MSV Rep domains.

Primer	**Mutagenic Primer**	**^{a}MSV Co-ordinates**
1 C1D^{99}**AYI**^{101}F	5'-AGAGTGAGGccTAttTTCTCAAGGAAC-3'	2092-2065
2 C1A^{99}**DYI**^{101}F	5'-AGAGTGAGGGATTACtTTCTCAAGGAAC-3'	2092-2065
3 C1D^{99}**AYI**	5'-AGAGTGAGGGccTATATTCTCAAGGAAC-3'	2092-2065
4 C1A^{99}**DY**^{100}FF^{101}I	5'-AGAGTGAGGGATTtTATTCTCAAGGAAC-3'	2092-2065
5 C1N^{200}**LE**^{201}K	5'-GATTTACTTTGTctTaAGTCAATCAAC-3'	1789-1763
6 C2Q^{248}PN^{249}Y	5'-CATAATTACTGGCcAtATAATGTTGATTG-3'	1553-1525
7 C2**K**^{234}H	5'-GCCCAAGTAGAaTgTCCGGTTCTTGTTG-3'	1570-1591

Primer	**Non-Mutagenic Primer**	**^{a}MSV Coordinates**
8 C1Univ1	5'-GTTAACTGACTTGGCACTCTG-3'	2093-2113
9 C1RbRev	5'-AGGTGAGGAGGGTGGATGAGG-3'	1790-1810
10 C2NTPRev	5'-AACCCCTAGGCTTCTGGCCC-3'	1554-1573
11 C2NTPR	5'-CAGAAGCCTAGGGGTTCATAATTACTGGCAAA-3'	1569-1538

Legend:

Bold uppercase letters denote conserved amino acids within the MSV Rep domain, lower case letters denote mutations, superscript numbers denote amino acid position, underlined letters; GG▼cc, *Hae*III; c▼tTaAG, *Bfr*I or *Afl*II; TGG▼CcA, *Msc*I engineered restriction sites. ^{a}MSV genome nucleotide positions starting at GGATCC.

amplified by PCR using primers C1 and C2 (designed by Dr Kenneth Palmer) which annealed to coordinates 2409-2386 and 1131-1153 of the Rep gene in the MSV-Kom genome. The primers had the following sequences: C1; 5'-ttaggATCCCTCAGCCTCAACCTC-3' and C2; 5'-ACGCAAACAATACAGGGGGGTAGT-3' where the lowercase nucleotides denote the 5' overhang mismatch that introduced a *Bam*HI restriction site g▼gATCC when the *rep* gene of MSV was amplified by PCR using the C1 primer as one of the primer pairs. PCR cycling conditions were: denaturing at 94°C for 2 min followed by 30 cycles at 94°C for

30 sec, 60°C for 30 sec, 72°C for 1 min and completion of the reactions at 72°C for 10 min. The PCR products were analysed by restriction with enzymes *Hae*III, *Bfr*I and *Msc*I whose sites were included as part of the mutagenic primer design. DNA for sequencing was isolated from the positive clones using the High Pure Plasmid kit (Boehringer Mannheim Corp., Indianapolis, IN, USA). Sequencing was performed on an ALFexpress automated DNA sequencer and the sequence data was processed by ALFwin version 1.1 software (Amersham Pharmacia Biotech AB, S-751 82 Uppsala, Sweden). All the sequence analyses were performed using the GCG program (Genetics Computer Group, program manual for the Wisconsin package, version 9.0, August 1996, GCG, 575 Science Drive, Madison, Wisconsin, USA 53711).

2.1.2 MSV Rep Nicking-Closing Motif III Primer Design

I designed the non-overlapping primers according to the requirements of the QuikChange^{TM} site-directed mutagenesis kit (Stratagene, La Jolla, CA USA). The primers varied in length ranging from 21 to 32 bases although the requirements specified a range of 25 to 45 bases. Four primers (1 to 4) and the abutting primer 8 in Table 2.1 were targeted to the amino acids of the nicking-closing motif III of MSV Rep characterised by VRD^{99}Y^{100}I^{101}LK. The sequence of the primers, the amino acid changes and annealing positions in the MSV-Kom genome are summarised in Table 2.1. The mutagenic primers 1 and 3 contained a *Hae*III restriction site GG▼cc to assist in selection of mutation carrying clones. Primer pairs 1, 8 and 3, 8 were used to amplify pKEP*rep*4 as outlined in Figure 2.1 to generate plasmids pTKS*rep*D^{99}AYI^{101}F and pTKS*rep*D^{99}AYI. The mutations were confirmed by sequencing (Figure 2.2, panels (b) and (e)) and plasmid, pTKS*rep*D^{99}AYI^{101}F was subsequently used as a template for PCR using primer pairs 2, 8 and 4, 8 (Table 2.1). The removal of the *Hae*III site was used for selecting the plasmids carrying mutations **Y**$^{100}\rightarrow$F and I$^{101}\rightarrow$F designated pTKS*rep*D**Y**^{100}FI and pTKS*rep*DYI^{101}F respectively (Figure 2.2 panels (c) and (d)). The non-distortion of the primer abutting site was confirmed by comparing all the mutation carrying plasmid sequences with the wild type (see Figure 2.2 panels (a) and (b)-(e)).

2.1.3 The Rb-binding Domain Abutting Primer Design

I designed two abutting primers for PCR (Table 2.1 primers 5 and 9) to change the MSV Rep Rb-binding domain amino acids N$^{201}\rightarrow$L and **E**$^{202}\rightarrow$K. The mutagenic primer 5, C1N^{201}**LE**^{202}K, contained an *Afl*II or *Bfr*I restriction site c▼tTaAG, which was used to select the mutant plasmid, pTKS*rep*N^{201}L**E**^{202}K. Plasmid pKEP*rep*4 was amplified by PCR as outlined in Figure 2.1. The DNA from putative clones was amplified by PCR using primers C1 and C2 as previously described and the products were cut with *Bfr*I. The mutations and primer-abutting site were confirmed by sequencing (Figure 2.2, panels (f) and (g)). The amino acid substitutions changed the Rb-binding domain from **LLCNE** to **LLCLK**.

2.1.4 The NTP-binding Domain ~~Abutting Primer Design~~

The MSV-Kom Rep putative NTP\ATPase-Helicase binding domain, **G**X$_4$**GKST**WARSX$_7$QNX$_{13}$NIV**DD**, was targeted for PCR site-directed mutagenesis of templates that already contained mutations to the nicking-closing motif, Rb-binding domain and / or both. The primers 6,10 and 7,11 in Table 2.1 were used to change the amino acids Q$^{248}\rightarrow$PN$^{249}\rightarrow$Y and **K**$^{234}\rightarrow$H. The mutagenic primer 6, C2Q^{248}PN^{249}Y, contained the *Msc*I restriction site TGG▾CcA for the selection of mutation-carrying plasmids. The putative clones were amplified by PCR using primers C1 and C2 and the products were cut with *Msc*I. The mutations and primer-abutting site were confirmed by sequencing (Figure 2.2, panels (h), (i)). The amino acids Q^{248} and N^{249} are not conserved residues of the NTP\ATPase-helicase domain: they are part of the sequence that determine the helicase activity of the Rep protein. I therefore designed a second primer pair, 7 C2$\mathbf{K}^{234}$H and 11 C2NTPR (Table 2.1) to change $\mathbf{K}^{234}\rightarrow$H. The amino acid $\mathbf{K}^{234}$ is conserved in the Rep proteins of replicons that constitute the superfamily (Gobalenya *et al.*, 1991). The mutagenic primer did not contain a restriction site because it was difficult to introduce one without changing the adjacent amino acids. I was unable to introduce the two mutations described as single mutant plasmids. Each time an unusual deletion of one or two nucleotides was obtained. This observation, for as yet unknown reasons, was confined to single mutant PCR products of the NTP\ATPase-helicase domain and absent from double or triple mutant combinations.

2.1.5 Computer Analysis of the Probable Secondary Structure of the Rep Mutant Proteins

The mutant and wild-type Rep gene sequences shown in Figure 2.2, panels (a)-(e), (g), (i) and various mutant combinations (sequences not shown) were analysed by a computer program that predicts the probable secondary structure of proteins according to Chou and Fasman (1978). The computer program is part of the Genetic Computer Group (GCG) suite (program manual for the Wisconsin package, version 9.0, August 1996, GCG, 575 Science Drive, Madison, Wisconsin, USA 53711). The proteins were analysed as products of full length spliced and unspliced C1:C2 mRNA, Rep and RepA, each having 355 and 272 amino acids respectively. The program also identified probable α-helices, β-pleated sheets, random coils and β-turns. The hydrophobic and hydrophilic regions were superimposed on the secondary structure.

2.2 RESULTS

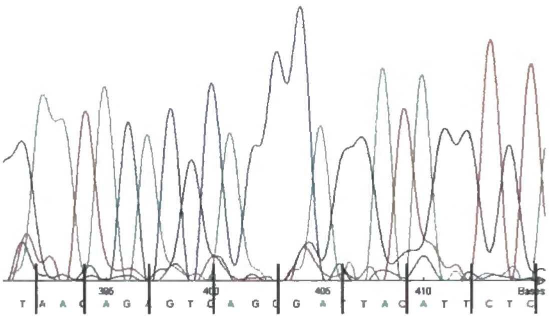
2.2.1 Comparison of MSV Rep Mutant and Wild-type Sequences

The sequence profile results (Figure 2.2 panels a-i) show plasmids carrying single mutations only, although various mutant combinations were produced. A total of nine plasmids carrying various mutations and combinations thereof in the Rep gene were generated using QuikChange site-directed mutagenesis. Four mutation-carrying plasmids were generated with mutations (3 single and 1 with amino acids flanking the conserved $\mathbf{Y}^{100}$) in the putative nicking-closing motif III of MSV-Kom Rep protein using primers described in Table 2.1. The sequenced clones were analysed to confirm the mutations and the non-distortion of the primer-abutting site as shown in Figure 2.2, panels (a-e). The clone containing the wild-type Rep gene was also sequenced and compared with the mutants as shown in Figure 2.2, panel (a). Sequence analysis and comparison with the wild-type Rep gene showed that *Pfu* DNA polymerase did not lead to imperfect primer abutting or displacement.

Two mutations were introduced into the Rb-binding domain, **LLCNE**, altering it to **LLCLK**. The mutations an~~d the primer-abutting site were confirmed by sequencing of~~ the plasmids selected after restriction with *Bfr*I (Figure 2.2, panels (f), (g)). There was no distortion or displacement of the abutting primers. In plasmids carrying the mutant Rb as part of a double or triple mutant combination (sequence results not shown), the abutting site was neither distorted nor displaced.

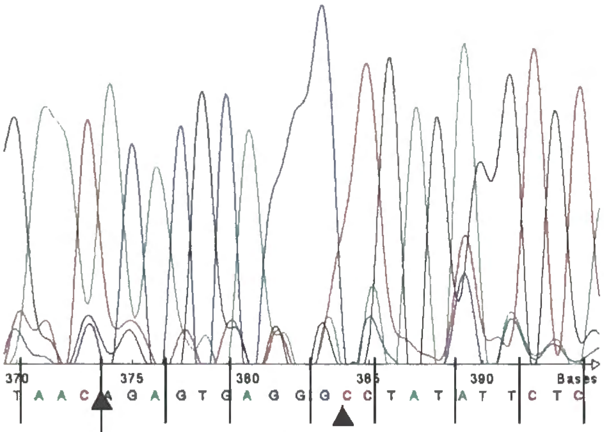
Two mutant plasmids, one containing two amino acid substitutions to the non-conserved helicase region of the Walker B loop and the other a single amino acid substitution to conserved **K**^{234} in the NTP-binding domain of the Walker A loop, were generated. The Walker A and B loops are typical of helicases and kinases (both are members of the superfamily) of plants and animals. The mutations and primer-abutting sites were confirmed by sequencing of the plasmids positively selected after restriction with *Msc*I. The sequences were compared with the wild-type Rep gene (Figure 2.2, panels (h), (i)). I was not able to produce both Q^{248}→PN^{249}→Y and **K**^{234}→H as single mutations although the mutations to variant amino acids were introduced as combinations of mutant motif III and the Rb-binding domain. It was also difficult to identify any clones carrying the **K**^{234}→H mutation using the T-tracking sequence approach.

(a)



Amino Acid Sequence ▶ N^{95} R V R D^{99} Y^{100} I^{101} L

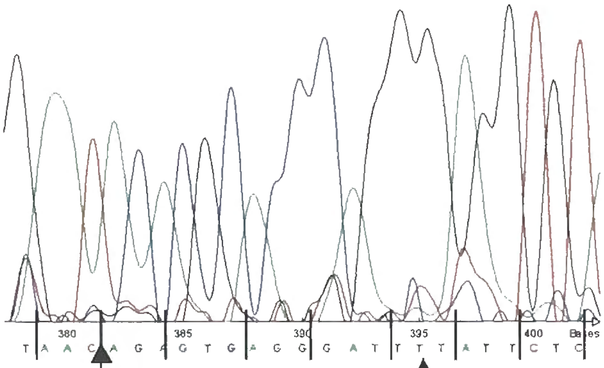
(b)



Amino Acid Sequence ▶ N^{95} R V R A^{99} Y^{100} I^{101} L

Abutting PCR primer site

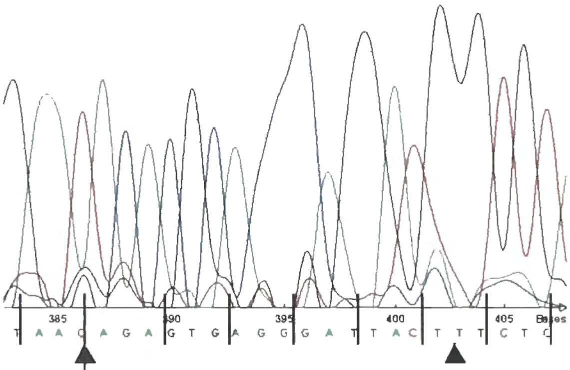
(c)



Amino Acid Sequence ▶N95 R V R D99 F100 I101 L

Abutting PCR primer site

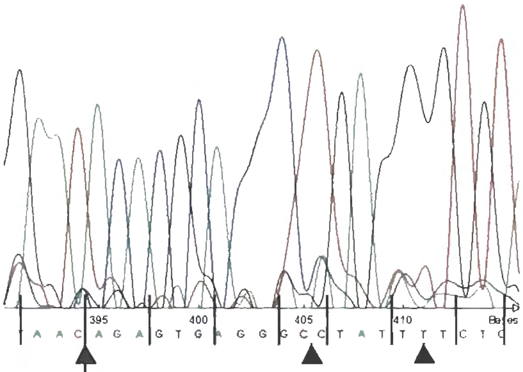
(d)



Amino Acid Sequence ▶N95 R V R D99 Y100 F101 L

Abutting PCR primer site

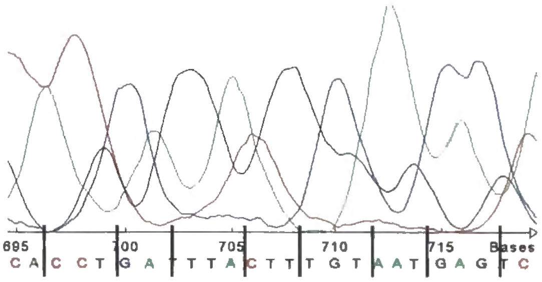
(e)



Amino Acid Sequence ▶N95 R V R A99 Y100 F L

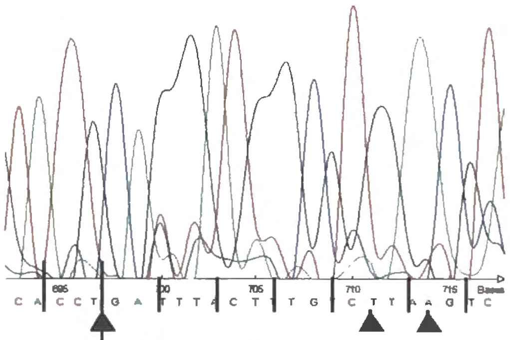
Abutting PCR primer site

(f)



Amino acid Sequence▶P^{196}D L L C N^{201}E^{202}

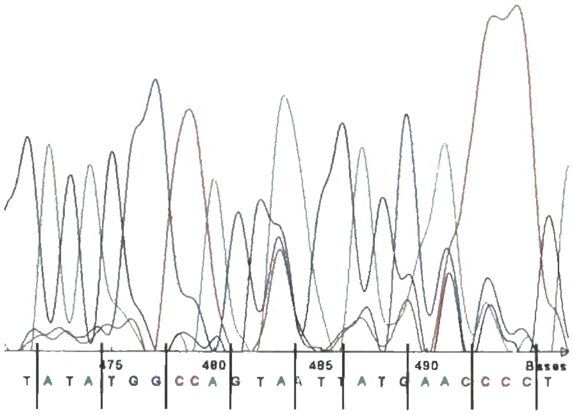
(g)



Amino acid Sequence▶P^{196}D L L C L^{201}K^{202}

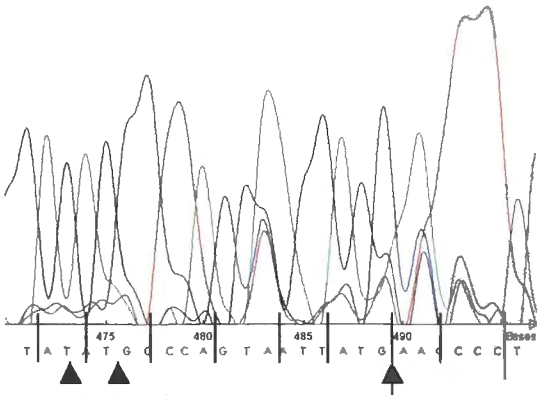
Abutting PCR Primers site

(h)



Amino Acid Sequence ▶ N^{249}Q^{248} W Y N H V G

(i)



Amino Acid Sequence ▶Y^{249}P^{248}W Y N H | V G

Abutting PCR Primers site

Figure 2.2. Comparison of mutant MSV Rep with wild type MSV Rep nucleotide sequences of functional domains. (a)-(e) MSV Rep sequences of the wild type, mutant D^{99}A, **Y**^{100}F, I^{101}F and D^{99}AYI^{101}F of the nicking-closing domain represented by amino acids N^{95}RVRA^{99}**Y**^{100}F^{101}L where the **Y** residue is conserved in all replicons which replicate via the rolling circle mechanism. (f), (g) MSV Rep sequences of the wild type domain P^{196}**DLLC**N^{201}**E**^{202} and mutated Rb-binding domain represented by amino acids P^{196}**DLLC**L^{201}K^{202} where **D** is a conserved aspartic acid residue that precedes the Rb-binding domain comprising conserved amino acid residues **L**, **C** and **E**. (h), (i) MSV wild type Rep sequences of the NTP and helicase domain represented by amino acids **G**X$_4$**GKSTW**ARSX$_7$Q^{248}N^{249}X$_{13}$NIV**DD** where **G**, **K**, **S**, **T** and **D** are conserved amino acids in the Rep proteins of geminiviruses. The mutated MSV Rep domain has a P^{248} and Y^{249} in place of Q and N respectively. The mutated domains are contained in various constructs used as described elsewhere in this thesis. The actual nucleotide changes are shown by arrow-heads and the primer abutting sites are shown by arrows. The sequences were obtained from printouts from the ALFexpress automated DNA sequencer (Pharmacia).

2.2.2 Predictions of the Two Dimensional Secondary Structure of the Amino Acid Sequences of Mutant Rep and Wild-type Rep ORFs

The secondary structure of a protein is defined by its local conformation, which is a function of the folding patterns of the polypeptide backbone: namely, helices, pleated sheets and turns. A polypeptide chain assumes a helical conformation if it is twisted to the same extent about each of its C_α atoms. Helices are held in place by hydrogen bonds. There are many types of polypeptide helices but for the purposes of this work the α-helix is the most important and is a common structural element of regulatory proteins, having an average of 11 amino acid residues per turn. The α-helix is a rigid arrangement of the polypeptide chain and is usually associated with hydrogen bonding within the chain, unlike β-pleated sheets. MSV Rep and RepA regulate the replication of MSV and the expression of late genes involved in the encapsidation and movement of the virus. RepA and / or Rep protein initiate replication of MSV in a topoisomerase I-like manner (Bisaro, 1996), pointing to its regulatory role.

α-helical structures are critical factors for homo- or hetero-dimerization of transcription regulatory proteins. In MSV-Kom Rep and RepA putative protein secondary structures generated from Chou and Fasman (1978) "plotstructures" (Figure 2.3), an α-helix is located between amino acids 32 and 43 (Hovarth *et al.*, 1998). The Chou-Fasman analysis of MSV-Kom Rep and RepA points to the presence of an identical hydrophobic region as reported for MSV-Nigeria Rep and RepA proteins. Horvath *et al.*, (1998) reported the presence of an amphipathic α-helix domain involving the 12 amino acids between 175 and 187 in both Rep and RepA proteins. An amphipathic molecule is simultaneously hydrophobic and hydrophilic. In MSV this domain is indispensable for the oligomerization of RepA and RepA-Rep proteins. Apart from the domains already described elsewhere in this thesis, RepA putatively has an antiparallel β-sheet between amino acids 225 and 230 and a β-sheet structure between amino acids 221 and 230. This region is also present in MSV-Kom and is different from known activation domains in that it is not acidic but has been shown to effectively activate transcription in MSV-Nigeria (Hovarth *et al.*, 1998). The MSV RepA activation domain is characterized by the amino acids, MLLQPTCYTL. The activation domain of MSV-Kom Rep starts within the NTP\ATPase-helicase domain between amino acids 252 and 270.

The results of the Chou and Fasman plotstructure analysis showed some interesting possible secondary structural changes due to mutations introduced into the conserved domains of MSV-Kom Rep and RepA. The predicted secondary structure has some interesting conformational changes (Figure 2.3 panel B and Bi compared with wild-type MSV-Kom Rep and RepA in panels A and Ai). The potential for radical changes in the conformation of Rep were probably due to the mutation $D^{99}\rightarrow A$ changing aspartic acid to alanine. Aspartic acid is a polar negatively charged amino acid whereas alanine is a tiny hydrophobic and non-polar amino acid. A change from a polar and hydrophilic to a non-polar and small amino acid could cause drastic structural changes of MSV-Kom Rep, as shown in Figure 2.3 panel B (compared with Figure 2.3 panel A). Suprisingly the same amino acid changes did not result in marked changes in the predicted structures of RepA (panel Bi) although changes were predicted within the Rb-binding domain (changes that compared very well with those in Figure 2.3 panel Fi). In Figure 2.3 panel B the Rb-binding domain of Rep did not change in contrast to RepA (panel Bi).

The most drastic changes predicted in Rep structure are from amino acids 250 and 355 (compare Figure 2.3 panel A with panel B). Mutating a tyrosine to a phenylalanine residue is regarded as a conservative change because the -OH group in tyrosine is replaced by an innate -H atom. The differences between the two amino acid residues are that tyrosine is polar whereas phenylalanine is non-polar. The predicted secondary structures of Rep and RepA carrying the mutation $Y^{100}\rightarrow F$ in Figure 2.3 (panels C and Ci) were not different from the wild type Rep and RepA (panels A and Ai). The potential for structural conservation with this conversion is confirmed by previously published work (Skiadopoulos and Faust, 1993; Hoogstraten *et al.*, 1996; Christopher *et al.*, 1990).

The predicted secondary structure of Rep and RepA carrying the mutation I^{101}→F resulted in increased hydrophilicity of the nicking-closing domain compared to all the other mutants (panels D and Di). Isoleucine is an aliphatic amino acid whereas phenylalanine is aromatic but, both are hydrophobic. The increase in hydrophilicity was probably due to the effect of adjacent amino acids. The mutations D^{99}→AYI^{101}→F in the nicking-closing domain resulted in potential conformational changes within the Rb-binding domain, and no predicted changes in the mutated domain (Figure 2.3 panels E and Ei). The potential conformational change was probably due to the changing of a charged polar aspartic acid (D) to a non-polar amino acid alanine (A) that has a methyl aliphatic hydrocarbon side chain. When the isoleucine flanking the conserved **Y**^{100} was changed to phenylalanine, this removed an isomeric butyl group and introduced a bulky aromatic group. The combined effect of these changes probably resulted in the changes predicted in Figure 2.3 panels E and Ei.

The predicted secondary structure resulting from mutating the Rb-binding domain is shown in Figure 2.3, panels (F) and (Fi) where a non-conserved asparagine (N) was changed to leucine (L) and the conserved glutamic acid (E) was changed to lysine (K). These mutations changed a polar amide-containing aspartic acid to leucine, a non-polar amino acid with an aliphatic isomeric butyl group. Glutamic acid is negatively charged whereas the basic amino acid lysine is positively charged. This probably caused the predicted conformational change from hydrophobic to hydrophilic (compare Figure 2.3, panels (A), (Ai) with (F), (Fi)).

The putative secondary structures of the double mutants of MSV Rep and RepA are shown in Figure 2.3, panels (G) and (Gi). The predicted conformational changes were the same as those previously described for panels (E), (Ei) and (F), (Fi). No additional changes to the predicted secondary structure were observed. These changes are probably conservative when introduced as double mutants in MSV Rep and RepA.

The NTP/ATPase-helicase domain is a component of MSV Rep alone, because the RepA-encoding ORF stops at amino acid 272 after the 94-bp intron that is spliced when MSV codes for the Rep protein. The predicted secondary structure of MSV Rep protein with the previously described mutations to the NTP/ATPase-helicase and Rb-binding domain is shown in Figure 2.3, panel (H). In the NTP/ATPase-helicase domain the non-conserved amino acids glutamine (Q) and asparagine (N) were changed to proline (P) and tyrosine (Y). These changes were interesting in that with the exception of proline, all the amino acids have a primary amino group and a carboxylic acid group substituent on the same carbon atom. Proline has a secondary amino group and is actually an α-imino acid. Changing any amino acid to proline would most probably result in some conformational changes because proline imposes conformational strains due to the cyclic nature of the pyrrolidine side group that is unique among the 20 amino acids. Remarkable possible conformational changes are shown in Figure 2.3, panel (I) from amino acid 240 and 270. This region also potentially becomes less hydrophobic, compared with panel (A) where the NTP/ATPase-helicase domain is wild type. The change from asparagine to phenylalanine is conservative because it involves changing from one non-polar amino acid to another, the difference being that asparagine has an amide-bearing side chain whereas phenylalanine has a phenyl moiety. The phenyl moiety is bulky but this did not result in a predicted increase in hydrophobicity, suggesting the importance of the introduced proline.

An interesting result was that of the predicted structure of the MSV Rep protein containing mutations to the nicking-closing and NTP/ATPase-helicase domains. In Figure 2.3, panels (E) and (Ei) mutations to the nicking-closing domain resulted in possible conformational changes that were also obtained in panels (F), (Fi), (G) and (Gi) where the Rb-binding domain only, and the nicking-closing and Rb-binding domains, were mutated. Although the contribution of individual mutated domains, that is the nicking-closing and Rb-binding domains in panel (G), could not be determined, it is reasonable to assume that the possible conformational changes in the Rb-binding domain were changed to wild type by mutating the NTP/ATPase-helicase (Figure 2.3, panel (I)). The conformational changes in the NTP/ATPase-helicase were as described for panel (H).

The MSV Rep protein carrying mutations to the three domains previously described has the same predicted conformation (Figure 2.3, panel (J)) as the one shown in panel (H). None of the MSV Rep and RepA plotstructures showed drastic conformational changes in the origin DNA-binding region that overlaps with the nicking-closing domain. In MSV Rep and RepA proteins the oligomerization domain between amino acids 175 and 187 does not overlap with the origin-binding region and was not predicted to change in any of the mutated proteins. Rep and RepA proteins support MSV replication as homo- or hetero-dimers and / or multimers.

The point of this exercise was to make constructs which would produce proteins which would interfere with MSV replication as "dominant negative" mutants. There are two possible mechanisms of resistance, one involving the association of mutated Rep or RepA with wild type Rep or RepA acting as inactive heterodimers or an inactive component of a multimer. The other interaction would be competition of the mutated and wild type protein for the MSV origin of replication. For these reasons all the constructs were used in the work described in subsequent chapters of this thesis.

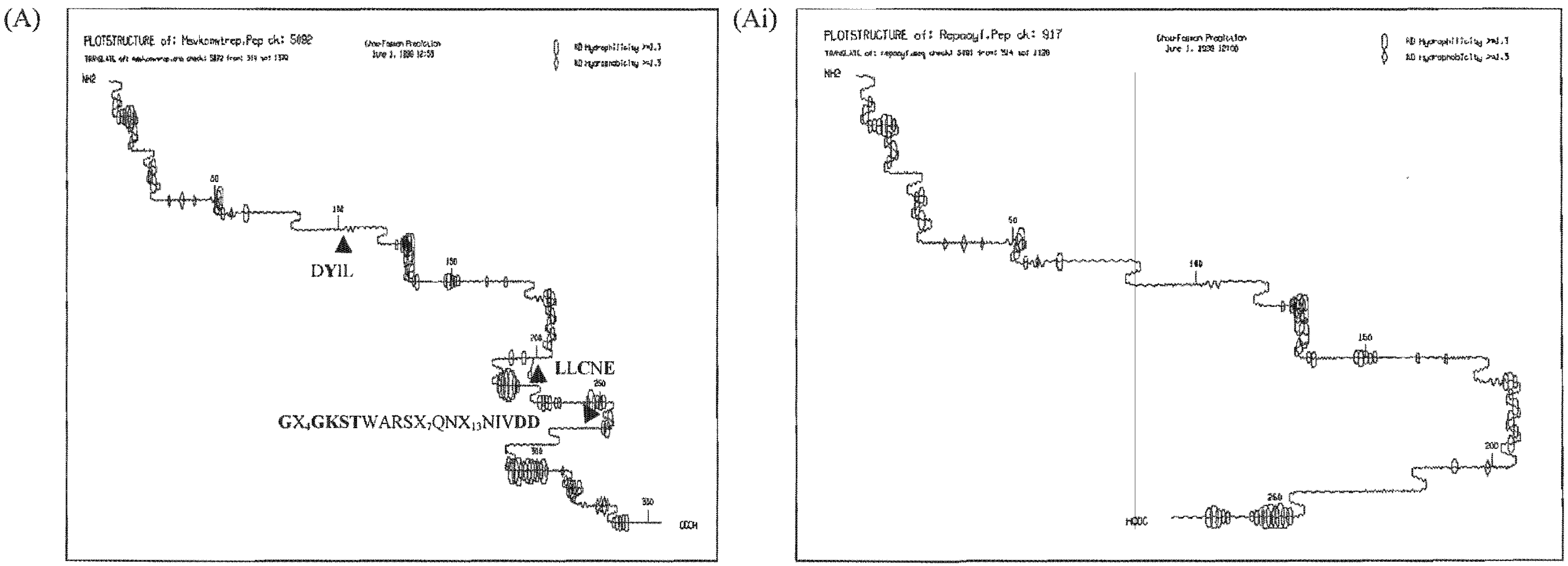


Figure 2.3. Predicted secondary structure, hydrophobicity and hydrophilicity patterns, of wild type and mutant MSV-Kom Rep and RepA proteins. The Chou-Fasman hydrophobicity and hydrophilicity of the proteins was set at >= 1.3. The predicted secondary structures of Rep and RepA spans amino acids 1-350 and 1-272 respectively. (A) Wild type MSV-Kom Rep protein, (Ai) wild type RepA protein.

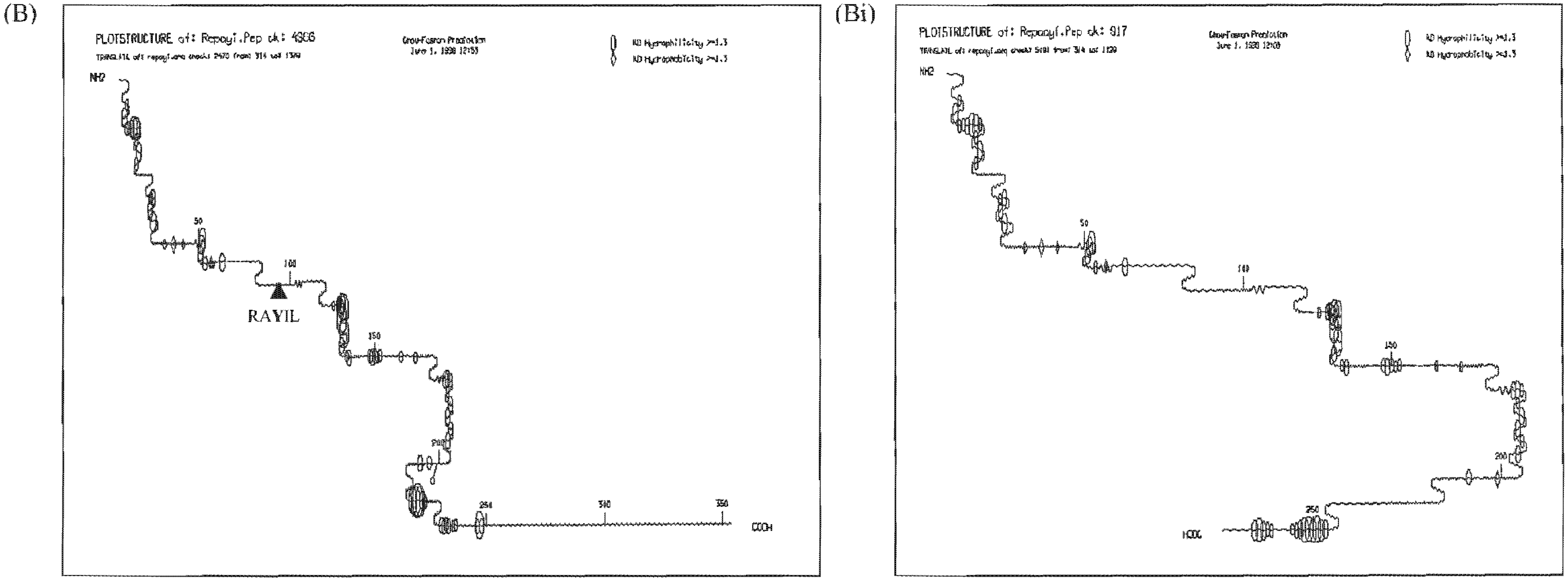


Figure 2.3/… (B) Mutant ($D^{99}A$) nicking-closing domain of MSV-Kom Rep, (Bi) mutant nicking-closing domain of MSV-Kom RepA.

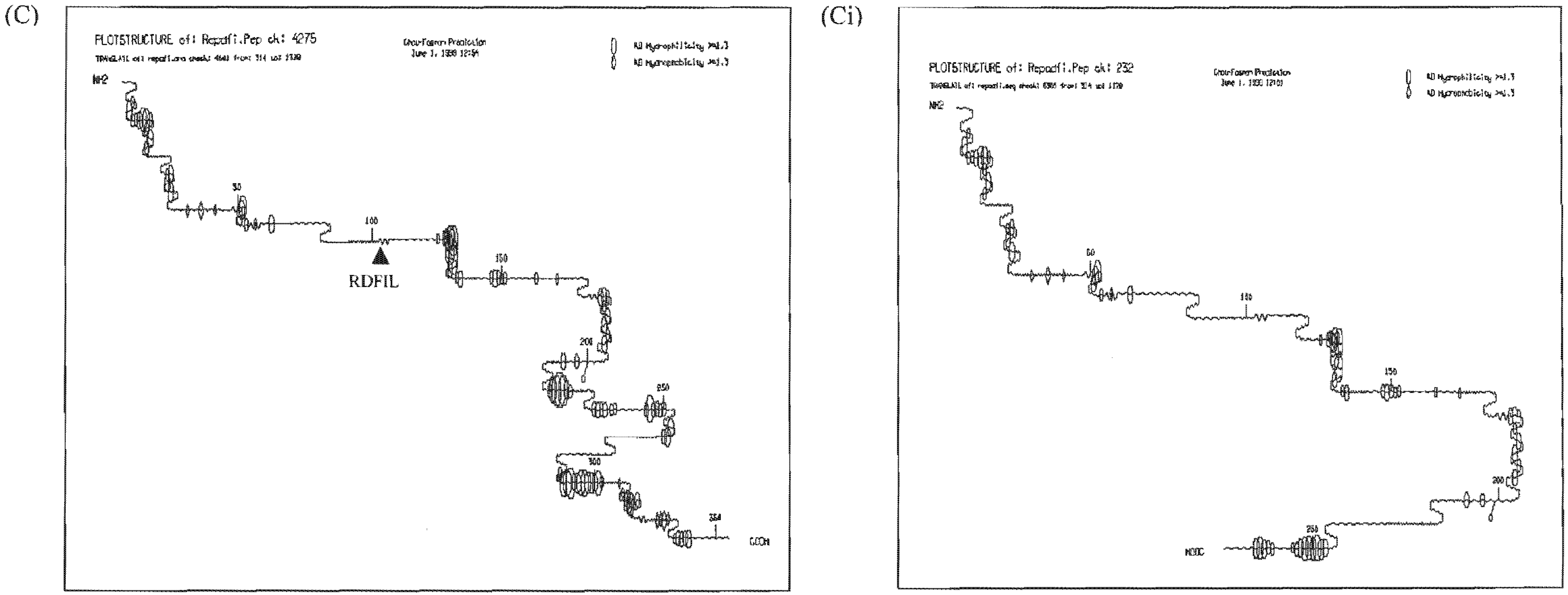


Figure 2.3/… (C) Mutant (Y^{100}F) nicking-closing domain of MSV-Kom Rep, (Ci) mutant nicking-closing domain MSV-Kom RepA.

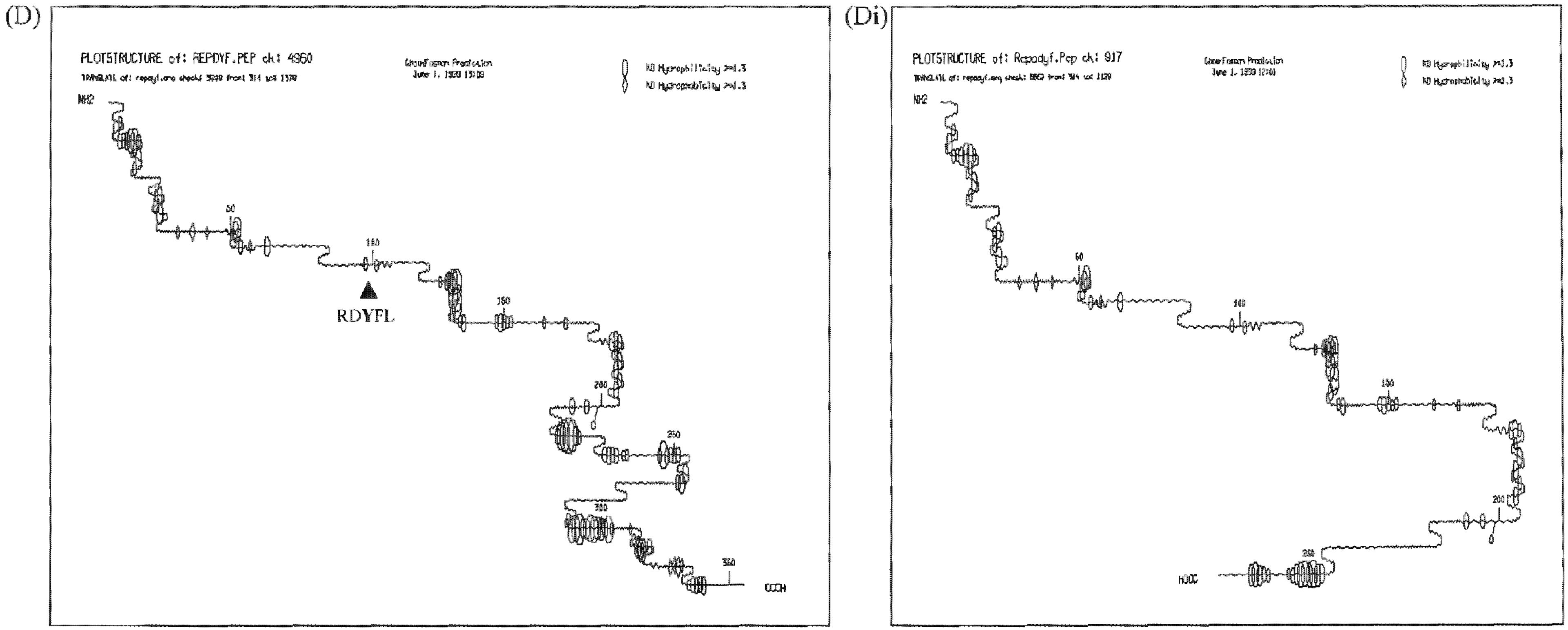


Figure 2.3/… (D) Mutant ($I^{101}F$) nicking-closing domain of MSV-Kom Rep, (Di) mutant nicking-closing domain of MSV-Kom RepA.

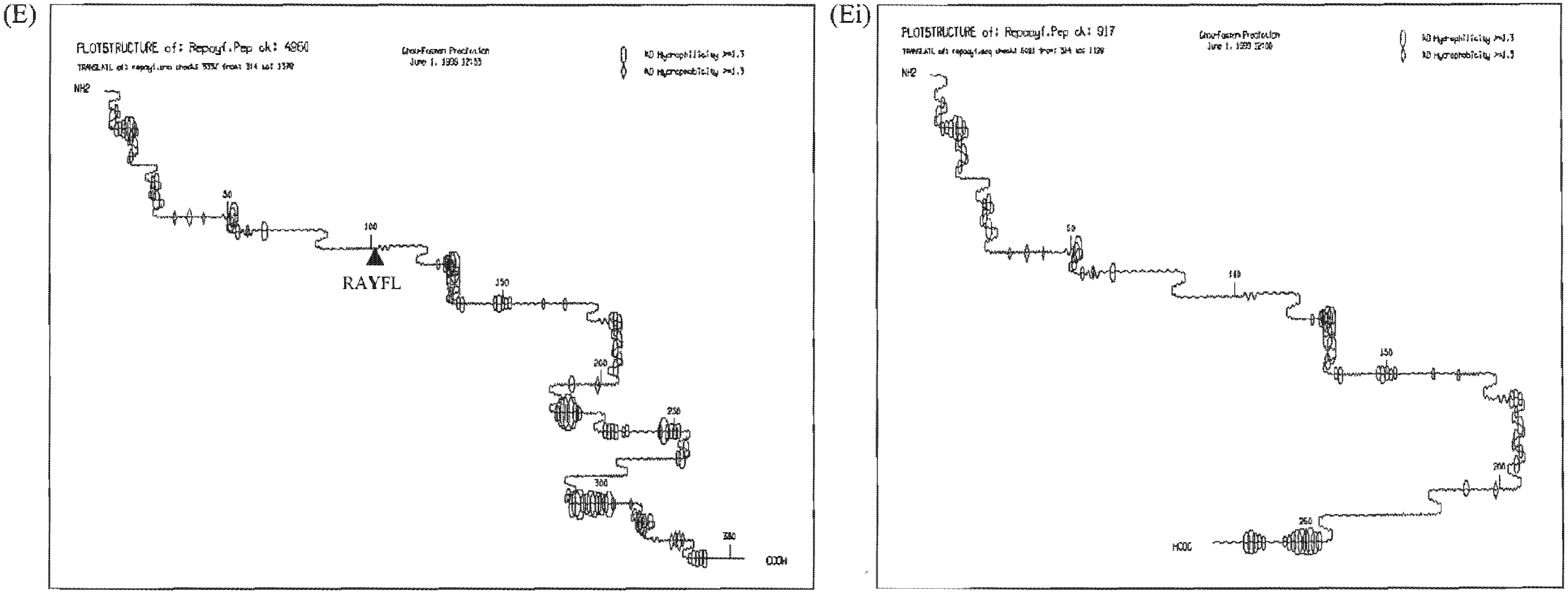


Figure 2.3/… (E) Mutant (D^{99}AYI^{101}F) nicking-closing domain of MSV-Kom Rep, (Ei) mutant nicking-closing domain of MSV-Kom RepA.

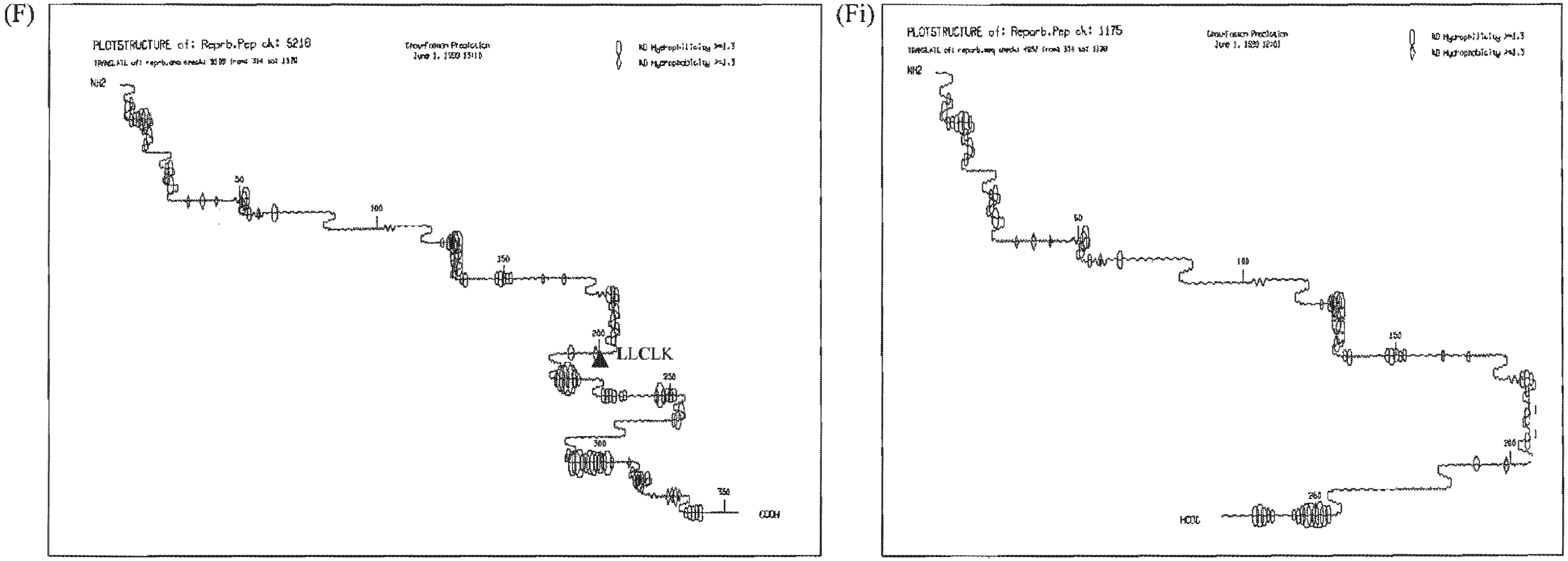


Figure 2.3/… (F) Mutant ($N^{201}LE^{202}K$) Rb-binding domain of MSV-Kom Rep, (Fi) mutant Rb-binding domain of MSV-Kom RepA.

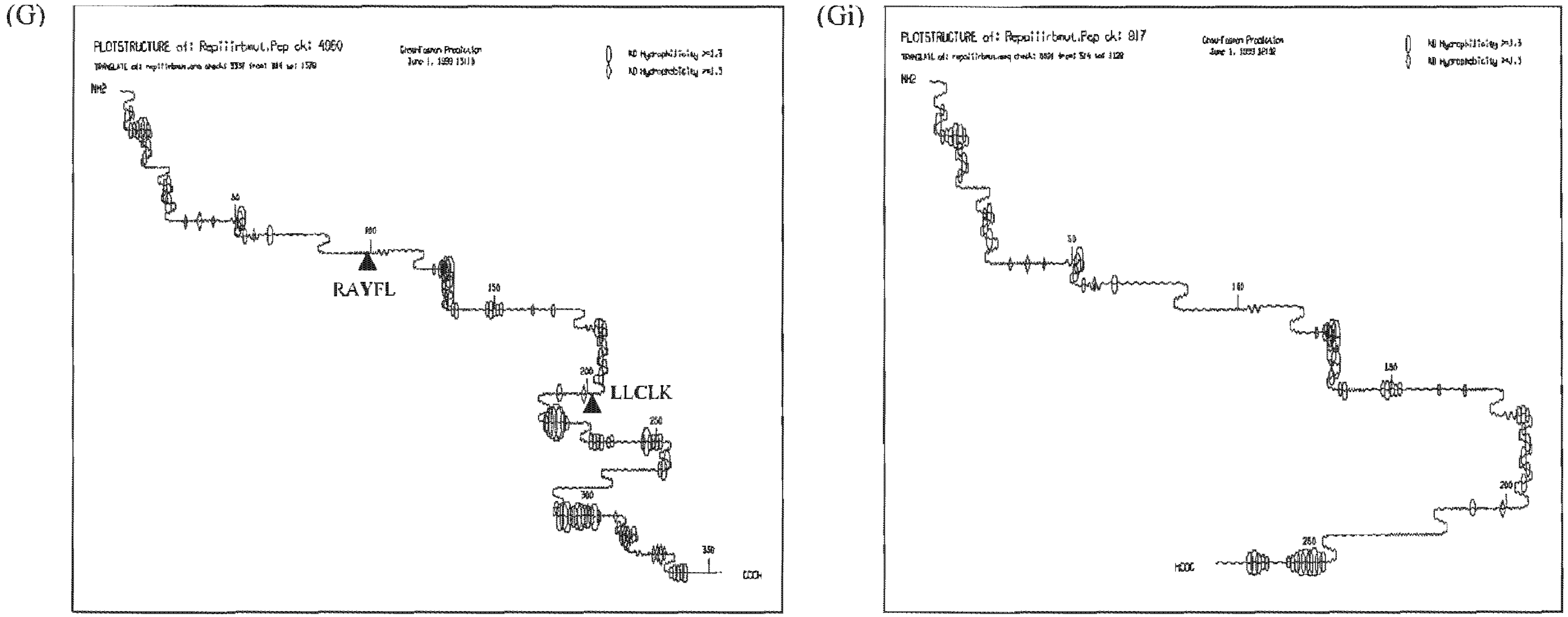


Figure 2.3/… (G) Double mutant (D^{99}AYI^{101}F) nicking-closing and (N^{201}LE^{202}K) Rb-binding domains of MSV-Kom Rep, (Gi) double mutant of MSV-Kom RepA.

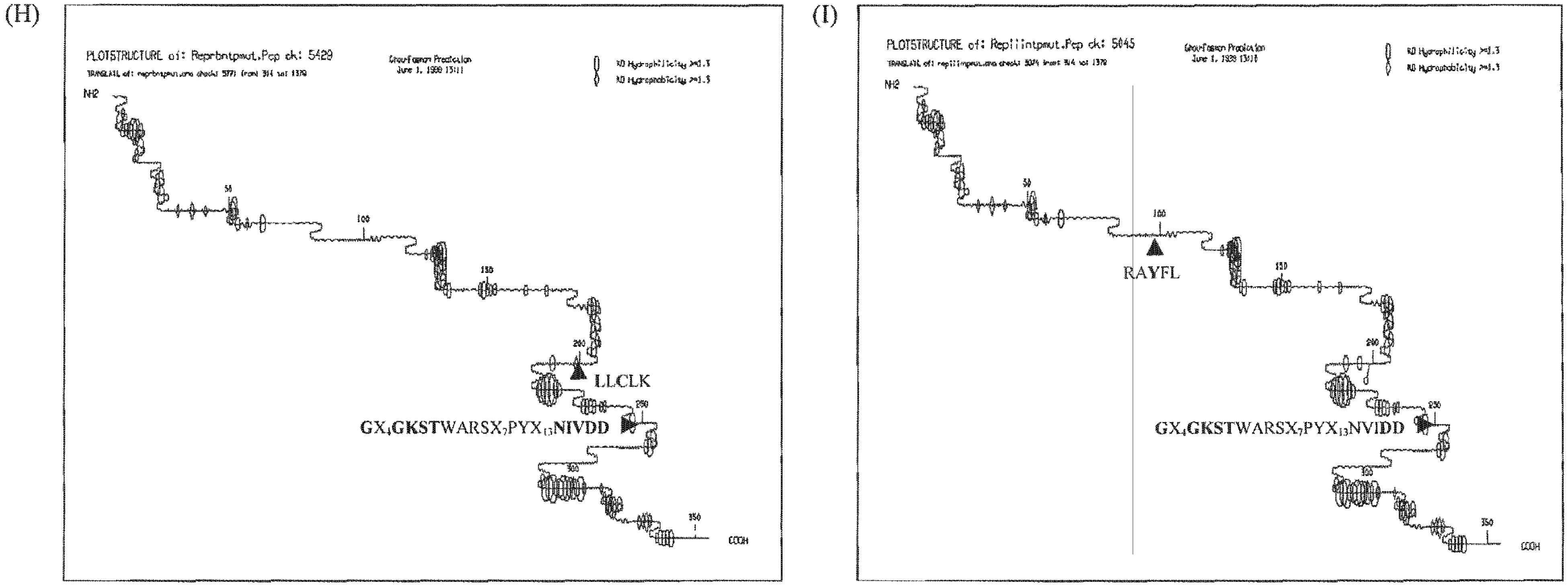


Figure 2.3/… (H) and (I), double-mutants ($N^{201}LE^{202}K$ and $Q^{248}PN^{249}Y$) and ($D^{99}AYI^{101}F$ and $Q^{248}PN^{249}Y$) of the MSV-Kom Rep domains previously described elsewhere in this chapter.

(J)

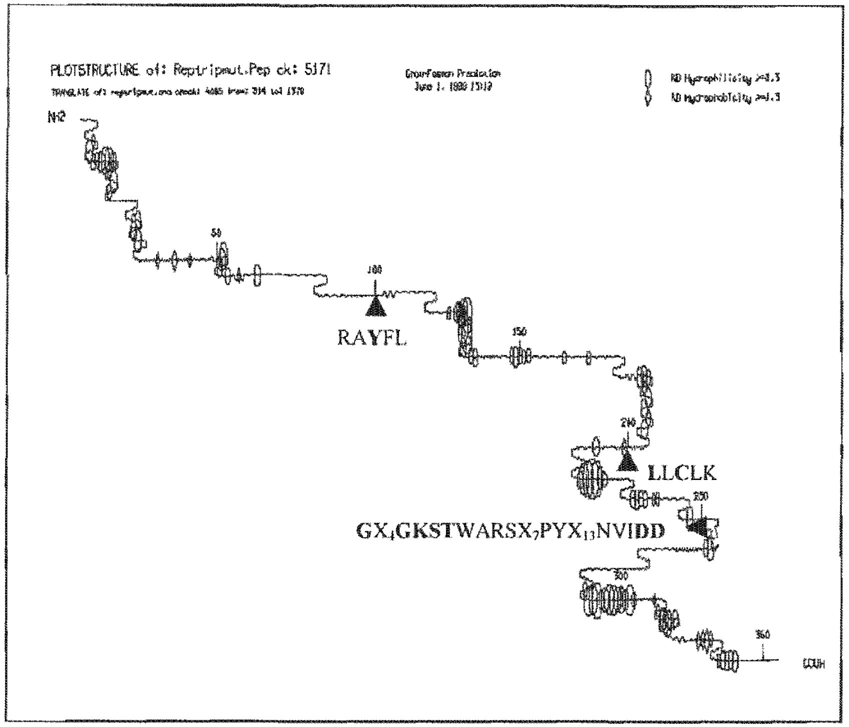


Figure 2.3. (J) Triple mutant containing all mutations to the three domains except that in the nicking-closing domain it contains the mutations shown in (E). Ovals denote hydrophilic regions and diamonds hydrophobic regions. The radius of the oval or diamond is proportional to the mean hydrophilicity or hydrophobicity calculated for that residue. The positions of α-helices are indicated as coils, β-sheets as narrow zigzags, β-turns as turns of the polypeptide chain and random coils as wide zigzags.

2.3 DICUSSION

PCR site-directed mutagenesis of MSV-Kom Rep was accomplished using a modified version of the methods of Briddon *et al.*, (1993) and Weiner *et al.*, (1994). Briddon *et al.*, (1993) demonstrated the use of AbP-PCR to produce infectious clones of ACMV in *Nicotiana benthamiana*. Modification of this method is the basis of most of the PCR site-directed mutagenesis methods described here. Undesirable mutations were obtained when the NTP/ATPase-helicase was targeted as a single domain mutant regardless of the enzyme being either *Pfu* or *Pwo* DNA polymerase. Currently there are no reports of either *Pfu* or *Pwo* adding or removing a 3'-end nucleotide as is known for *Taq* DNA polymerase. I did not manage to produce desirable mutants using *Pwo* DNA polymerase. The clones either contained an inadvertent deletion or insertion of a nucleotide. The deletions may be a result of the ligation of incomplete extension products as previously reported for the PCR site-directed mutagenesis of the human EST1 gene (Fisher and Pei, 1997) or of primers a nucleotide shorter than the one designed. As yet, there are no known reports of *Pwo* or ordinary *Taq* DNA polymerase removing a 3'-end nucleotide although *Pwo* has 5' exonuclease activity that accounts for its high fidelity. A combination of *Pfu* DNA polymerase and *Dpn*I digestion of ligation PCR products resulted in a marked increase of the desired mutations. A combination of heat tolerance and 3'→5' exonuclease activity make DNA polymerases, *Pfu* and *Pwo* enzymes of choice for PCR site-directed mutagenesis.

The non-distortion of the abutting primer sites (a probable site of undesirable mutations due to imperfect abutting of primers) were checked by sequencing the MSV-Kom Rep clones. None of the selected clones contained any distortion or undesirable mutations or deletions (Figure 2.2 a-i). For as yet unknown reasons I failed to produce single domain mutants in the NTP/ATPase-helicase domain. There was consistent addition or removal of a nucleotide in all the clones that were sequenced.

The secondary structure of the various mutants was predicted using the Chou-Fasman program as was reported earlier (Beetham, 1997; Hovarth *et al.*, 1998). The design of the MSV-Kom Rep or RepA mutants was based on disrupting some of the functions of the protein whilst keeping aspects like origin recognition, binding and oligomerization intact. In TGMV the AL1 protein forms large multimeric complexes (Orozco *et al.*, 1997) probably through oligo- or heterodimerization. In MSV-Nigeria experiments have demonstrated homo- and heterooligomer formation between RepA and Rep (Hovarth *et al.*, 1998). The Rep or RepA protein has regulatory properties like plant and animal transcription factors. It is therefore reasonable to assume that the inter- or intramolecular interactions between Rep or RepA can affect the binding and specificity of the monomeric or homodimeric forms of the proteins. In TGMV, DNA binding by the AL1 protein was dependent on AL1/AL1 interaction as opposed to nicking and closing that did not require AL1/AL1 interaction (Orozco and Hanley-Bowdoin, 1998). The interactions between Rep and RepA were used as a rationale for using MSV-Kom Rep ORF with the intron and the introduction of mutations in the functional domains described elsewhere in this thesis.

A number of mutations were generated in motif III because of earlier reports that delineated the functions of the conserved tyrosine residue in the TGMV Rep (Orozco and Hanley-Bowdoin, 1998). The aromatic ring of the conserved tyrosine of TGMV Rep is essential for binding while the hydroxyl group is necessary for DNA cleavage. The change of tyrosine to phenylalanine keeps the aromatic ring intact while changing the hydroxyl group. This change will probably not result in extensive conformational changes (see Figures 2.3 C and Ci; A and Ai). The changes, if any, are very conservative and it is reasonable to predict that if the mutant is expressed in a plant vector it will result in competition leading to a resistance phenotype. The amino acid residues flanking the conserved tyrosine when changed to alanine and phenylalanine respectively, could result in potentially drastic and mild structural changes respectively (see Figures 2.3 A and Ai compared to B and Bi; D and Di respectively). The mutant in Figure 2.3 B and Bi when expressed could result in inefficient binding and nicking-closing because of the conformational changes. The increase in hydrophilicity (see Figure 2.3 D and Di) would probably have a negative impact on the binding competitiveness of the mutant protein. The nicking-closing capability can not be inferred from the predicted secondary structures. The double mutant (see Figure 2.3 E and Ei) did not result in noticeable conformational changes except in the Rb-binding domain. What can not be deduced from this increase in hydrophobicity is whether it will lead to a decrease in DNA binding or not.

The double mutant (see Figure 2.3 F and Fi) did not lead to any additional predicted changes other than those described for the nicking-closing double mutant (see Figure 2.3 E and Ei). The full-length Rep or RepA is reasonably conserved to warrant the assumption that it will perform the basic function of binding competitively. The various mutant combinations did not result in extensive predicted conformational changes except where the NTP\ATPase-helicase domain was changed (see Figures 2.3 H, I, and J).

The mutations that I introduced were outside the oligomerization domain (amino acids 175 to 187), it is therefore reasonable to assume that the mutants will lead to “multistage” resistance when expressed in plants. As splicing is an important aspect of gene expression, the use of full-length Rep ORF probably result in the differential splicing of the intron in different maize genotypes and during different plant developmental stages. The oligomerization domain in both RepA and Rep facilitate the following interactions: RepA↔Rep*, RepA*↔Rep, Rep*↔Rep and RepA↔Rep* that are non-productive (where * is a mutant, described elsewhere in this thesis). The "multistage" resistance is due to the formation of dysfunctional homo-, hetero- and multimers, competition between wild type and mutant Rep or RepA for the MSV-Kom origin of replication and the level of Rep*, Rep, RepA and RepA* (vis-à-vis their effect (if any) on splicing).

CHAPTER THREE

CONTENTS

CHAPTER THREE

Mutational Analysis of the Conserved Domains in the Replication Initiation Protein (Rep) of MSV

ABSTRACT

The Rep amino acid substitutions D^{99}→A, $\mathbf{Y}^{100}$→F and I^{101}→F of the nicking-closing motif, and Q^{248}→P and N^{249}→Y of the NTP\ATPase-helicase domain of MSV-Kom were studied. Changes to the variable amino acids D^{99}→A, I^{101}→F, Q^{248}→P, N^{249}→Y and invariant amino acid $\mathbf{Y}^{100}$→F, failed to support MSV-Kom replication and agroinfection. The results of agroinfection and replication assays demonstrate that the variable amino acids flanking the conserved $\mathbf{Y}^{100}$ and those in the NTP\ATPase-helicase domain are also essential for MSV-Kom replication, infectivity and symptom development. It has been reported that RepA and not Rep of the dicot mastrevirus BeYDV, binds to the maize retinoblastoma (Rb) protein via the consensus binding motif **LxCxE**. In MSV the Rb-binding domain, LLC$N^{201}\mathbf{E}^{202}$ was mutated to **LLCLK**. The plasmid carrying N^{201}→L and $\mathbf{E}^{202}$→K as single-domain mutations supported replication and complemented replication-incompetent MSV-Kom in BMS cells. Moreover, a mutant viral construct was agroinfectious in susceptible Jubilee sweetcorn plants. The results demonstrate that in rapidly dividing cells the Rb-binding domain is not essential, and that it can tolerate amino acid changes *in planta*.

3.0 INTRODUCTION

MSV is transmitted into the phloem sieve tubes of the host plant by *Cicadulina mbila*, limiting the virus invasion to phloem-associated cells such as bundle sheath and mesophyll cells (Pinner *et al.*, 1996). MSV, like other maize geminiviruses, does not invade the shoot apical meristem of maize favouring a vascular limitation that is lost in tissues of mature leaves (Lucy *et al.*, 1996). Geminiviruses do not encode DNA or RNA polymerases for replication and transcription; they rely on the host cellular machinery. For successful infections, MSV must be competent in recruiting host cell factors and co-ordinating the virus-host cell cycles.

There is good experimental evidence for small DNA genome viruses initiating virus-host protein interactions that create a permissive environment for virus replication. This interaction has been well studied in the small animal DNA tumour virus, SV40, where the large T-antigen interacts with proteins of cellular origin to enable viral DNA replication and transcription (Fanning and Knippers, 1992). In the case of MSV, it is known that maize nuclear factors interact with LIR sequence elements responsible for the rightward expression of virion-sense genes (Fenoll *et al.*, 1988; 1990). Accotto *et al.* (1993) demonstrated that the accumulation of double stranded forms of the mastrevirus DSV was correlated with the S-phase of mitosis in *Digitaria setigera* cells. However, the replication of WDV in BMS cells and in non-dividing protoplasts from maize endosperm tissue culture apparently ~~did not show preference for particular phases of the cell cycle~~ (Timmermans *et al.*, 1992). In the latter case, this may be due to the cells being differentiated and vegetative; however, this was not investigated. The third piece of evidence is the induction of the proliferating cell nuclear antigen (PCNA), a marker for

DNA synthesis and progression through the S-phase, by infection with the begomovirus TGMV, or by constitutive expression of TGMV Rep, in quiescent terminally differentiated leaf cells of *Nicotiana bethamiana* (Nagar *et al.*, 1995). The fourth and probably most interesting stage is the interaction of the RepA protein, shown for WDV in yeast, with p130^{Rbr2}, a human Rb-like protein involved in the control of cell cycle progression (Xie *et al.*, 1995).

One of the critical stages in the life cycle of MSV is the regulation of the expression of RepA or Rep by splicing of the C1:C2 ORF. Splicing is an important gene regulation mechanism in plants that is associated with organ-specific and developmental stage-specific gene expression (Gorlach *et al.*, 1995; Hirose *et al.*, 1993; Kopriva *et al.*, 1995; Rundle and Zielinski, 1991). The coupled MSV invasion of various tissues and the emergence of new leaves from the whorl in maize suggest that MSV gene regulation by splicing may be important (Lucy *et al.*, 1996). In MSV infected maize 80% of the complementary-sense transcripts produce RepA while 20% produce Rep (Wright *et al.*, 1997). Similar splicing has been shown for TYDV, WDV and DSV.

Both RepA and Rep of MSV are multifunctional proteins with modular functional domains. Although mutational analysis has been done to determine the functions of the movement (MP) and coat (CP) proteins of MSV-N (Boulton *et al.*, 1991), not much has been done to evaluate the importance of individual amino acids within the functional domains of the complementary-sense gene products. Instead, the functional regions such as the α-helices, nicking-closing, Rb-binding, oligomerization and NTP\ATPase-helicase motifs or domains of MSV-Nigeria RepA and Rep were predicted and demonstrated by protein-protein interaction analysis using the yeast two-hybrid system (Horvath *et al.*, 1998). Mutational analysis of the conserved nicking-closing, Rb- and NTP\ATPase-helicase motifs with the intention of producing resistant plants has not been much investigated in mastreviruses. Mutations to the nicking-closing motif have been studied in bacterial plasmids, pC194, pT181 and pMV158 (Noirot-Gros *et al.*, 1994; Christopher *et al.*, 1990; Moscoso *et al.*, 1997), minute viruses of mice, and the begomoviruses BGMV and ACMV (Hoogstraten *et al.*, 1996; Stanley, 1995). Mutational analysis of dicot infecting BeYDV ORFs demonstrated the functional conservation of equivalent genes in mastreviruses that infect monocotyledonous plants (Liu *et al.*, 1998). In BeYDV the Rb binding domain tolerated mutations in the consensus **LxCxE** motif (Liu *et al.*, 1999). The **L**→I, **C**→S, **C**→G and **E**→Q mutants to the **LxCxE** of BeYDV were able to replicate in tobacco protoplasts and to systemically infect *Nicotiana bethamiana* and bean. However, the **LxCxE** mutants, **C**→G and **E**→K of WDV did not replicate efficiently, or failed to replicate in wheat suspension cells (Xie *et al.*, 1995).

The NTP\ATPase-helicase domain is commonly found in kinases and DNA helicases (Gorbalenya *et al.*, 1990). The Rep protein of geminiviruses belongs to the helicase superfamily III that includes proteins like the SV40 large T-antigen and NS1 of parvoviruses (Gorbalenya *et al.*, 1990). This domain is involved in the unwinding of replicating DNA templates. Mutational analysis of the conserved amino acids $\mathbf{EGX_4GKSTX_{32}N}I\mathbf{VDD}$ demonstrated that the domain was essential for BGMV replication (Hanson *et al.*, 1995).

I report studies on the mutational analysis of the nicking-closing, Rb-binding and NTP\ATPase-helicase domains in the RepA or Rep of MSV-Kom in cultured BMS suspension cells and maize plants. I used single mutants, all combinations of double mutants, and triple mutants of the functional domains of MSV-Kom. In addition, the possible use of these mutants to develop resistant transgenic maize is discussed.

3.1 MATERIALS AND METHODS

3.1.1 Construction of Rep Mutants of MSV-Kom

The sequenced mutants of the Rep gene, cloned in the *Bam*HI-*Eco*RV site of pKS, were used to introduce mutations of the conserved Rep domains into the replicative MSV-Kom construct pKom602 ((a gift from Dr W Schnippenkoetter); Figure 3.1). The mutations in the Rep gene were introduced into pKom602 on a 1.0-kb *Bgl*II-*Nsi*I fragment which replaced the wild type Rep gene fragment. The MSV-Kom mutant plasmids were designated pTK*rep*$^{\mathrm{Mut}}$. They were used to investigate the effect of the mutations on the replication of MSV-Kom in rapidly dividing BMS cells.

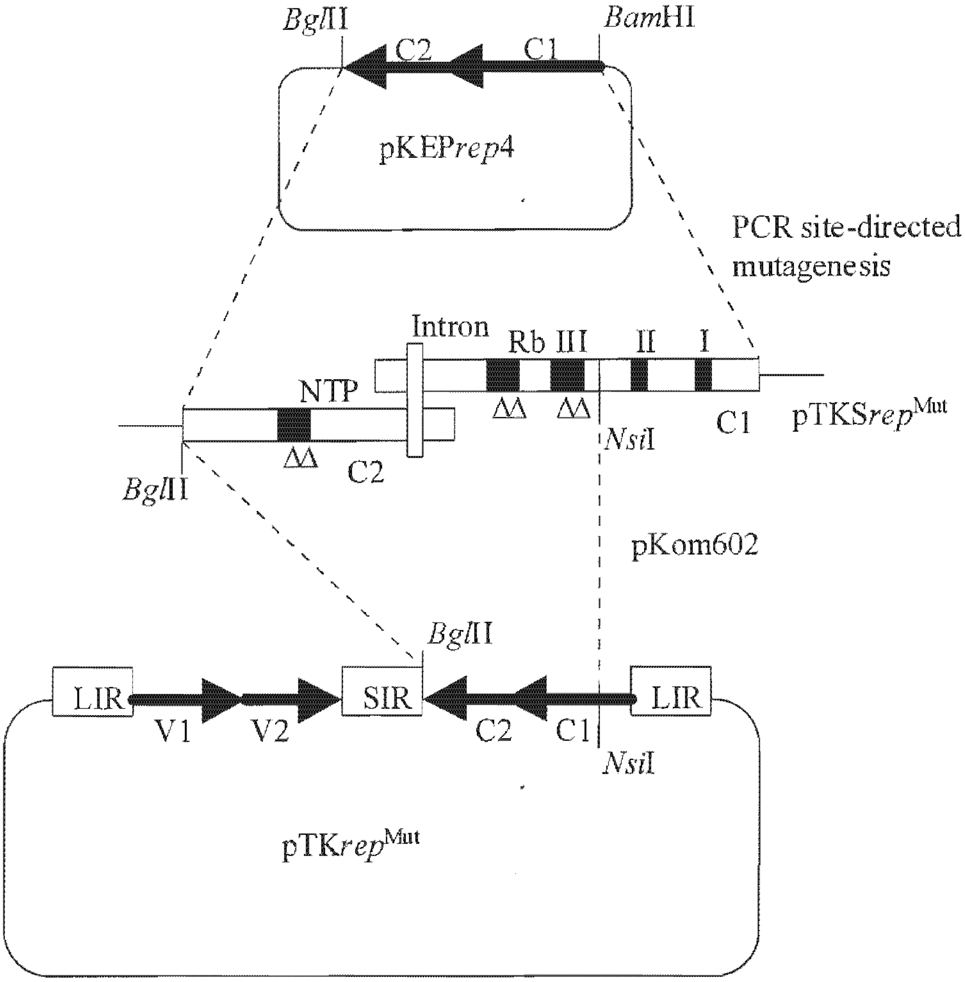


Figure 3.1. Schematic representation of the MSV-Kom *rep* wild type gene replacements with the *rep* fragment containing the various mutations. Abbreviations: C1 and C2, the complementary sense genes of MSV-Kom; I, II, III, Rb, NTP, domains described in Table 3.1; Δ, a point mutation introduced by AbP-PCR site-directed mutagenesis (see Figure 2.1).

3.1.2 Construction of MSV-Kom Rep Mutant Constructs for *in planta* Replication Analysis

An *Xba*I-*Eco*RI 1.1-mer fragment excised from pTK*rep*^{Mut} or pKom602 was ligated into *Xba*I-*Eco*RI digested plasmid pBI121 (CLONETECH Laboratories, Inc., California USA), an *Agrobacterium* binary vector, resulting in vectors pTB*rep*^{Mut} or pTB*rep*$^{\mathrm{Wt}}$ (Figure 3.2). The constructs were used to investigate the effect of the mutations on the replication of MSV-Kom *in planta* and the infectivity of MSV-Kom carrying the various mutations.

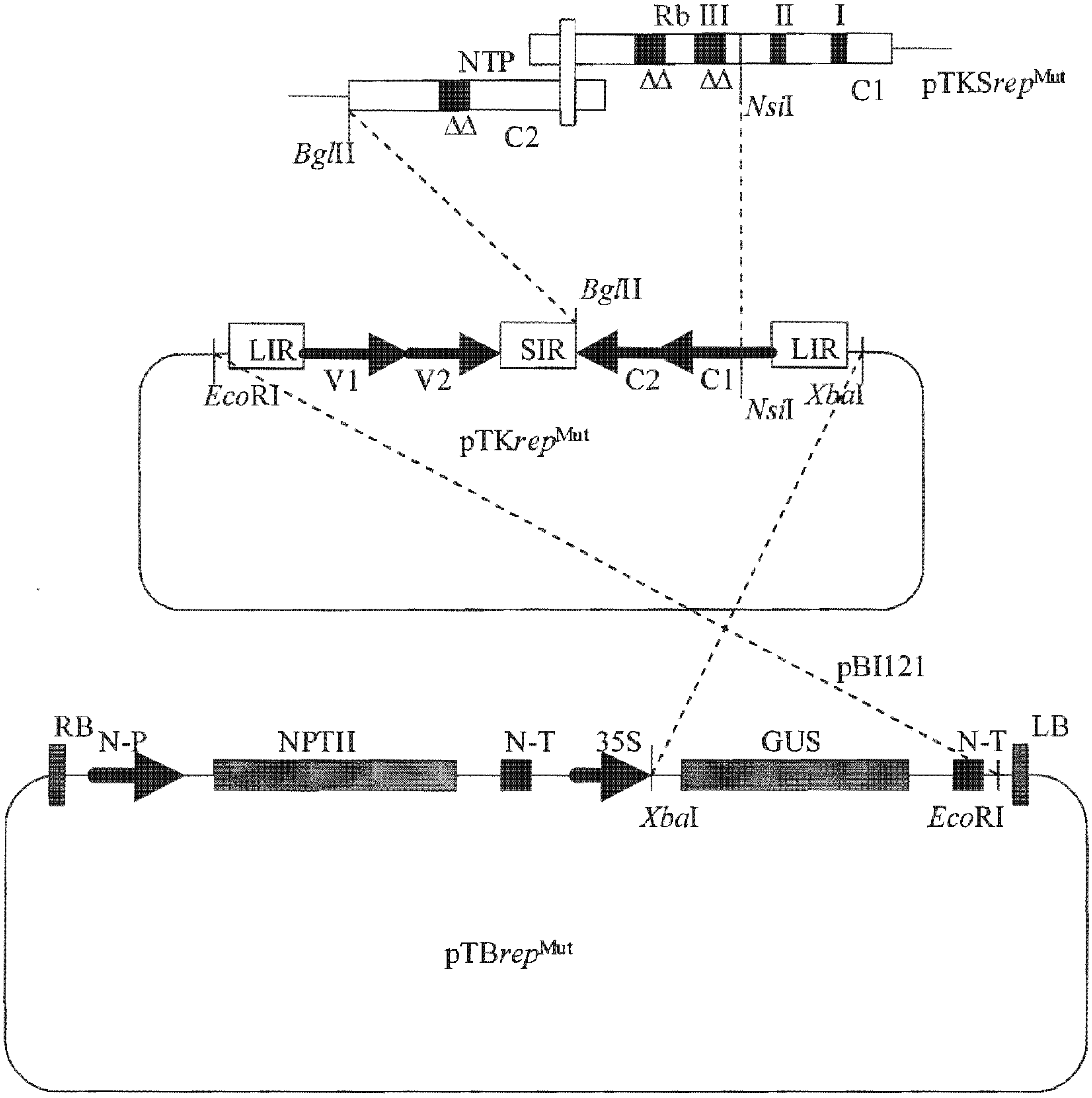


Figure 3.2. A schematic representation of the binary vectors containing the MSV-Kom Rep gene carrying mutations in the conserved domains. pTB*rep*$^{\mathrm{Mut}}$ is derived from pIBI121 with the GUS gene replaced by a 1.1-mer from pTK*rep*$^{\mathrm{Mut}}$. The pTB*rep*$^{\mathrm{Mut}}$ contains a NPTII (kanamycin resistance) gene 3' of the N-P (Nos-promoter) and 5' of the N-T (Nos-terminator). It also contains a 1.1-mer carrying a mutated MSV-Kom Rep gene. All these sequences are contained between the right border (RB) and left border (LB).

Instead of analysing the effect of individual mutations within the nicking-closing motif III on MSV-Kom replication in BMS cells, the analysis was done *in planta* making it necessary to clone the 1.1-mer MSV-Kom in a binary vector. Individual mutations to the amino acids of the nicking-closing motif were dealt with separately in this Chapter. The *Xba*I-*Eco*RI fragments from constructs; pTK*rep*$^{\mathrm{D99AYI}}$, pTK*rep*$^{\mathrm{DY100FI}}$ and pTK*rep*$^{\mathrm{DYI101F}}$ constructed as described elsewhere in this Chapter were each cloned into the *Xba*I-*Eco*RI site of the *Agrobacterium tumefaciens* binary vector pBI121 (Figure 3.2). The resultant plasmids; pTB*rep*$^{\mathrm{D99AYI}}$, pTB*rep*$^{\mathrm{DY100FI}}$ and pTB*rep*$^{\mathrm{DYI101F}}$, transformed into *A. tumefaciens*, were used for *in planta* evaluation of infectivity using pTB*rep*$^{\mathrm{D99AYI101F}}$ (this Chapter) as a negative control.

3.1.3 Construction of Plant Vectors for *trans*-Replication Analysis of Mutant Rep Proteins

A full-length 1.3-kb *Bam*HI-*Bgl*II fragment of wild type and mutant Rep gene was cloned into the *Bam*HI site of 5' dephosphorylated plasmid, pAHC17 (Carnejo *et al.*, 1993). Plant vectors with the Rep gene in the sense orientation were selected for further use. The resultant constructs were designated pTA*rep*$^{\mathrm{Mut}}$ (Figure 3.3). pTA*rep*$^{\mathrm{Wt}}$, containing the wild type Rep gene in the same orientation, was used as a control in the replication assays. To ensure that the pTA*rep*$^{\mathrm{Mut/Wt}}$ constructs were expressing the gene product of interest, complementation experiments were performed using replication-incompetent MSV-Kom, pKEP177-*Pst*I or p35S-GUSLSIR (Palmer, 1997; Willment, 1999).

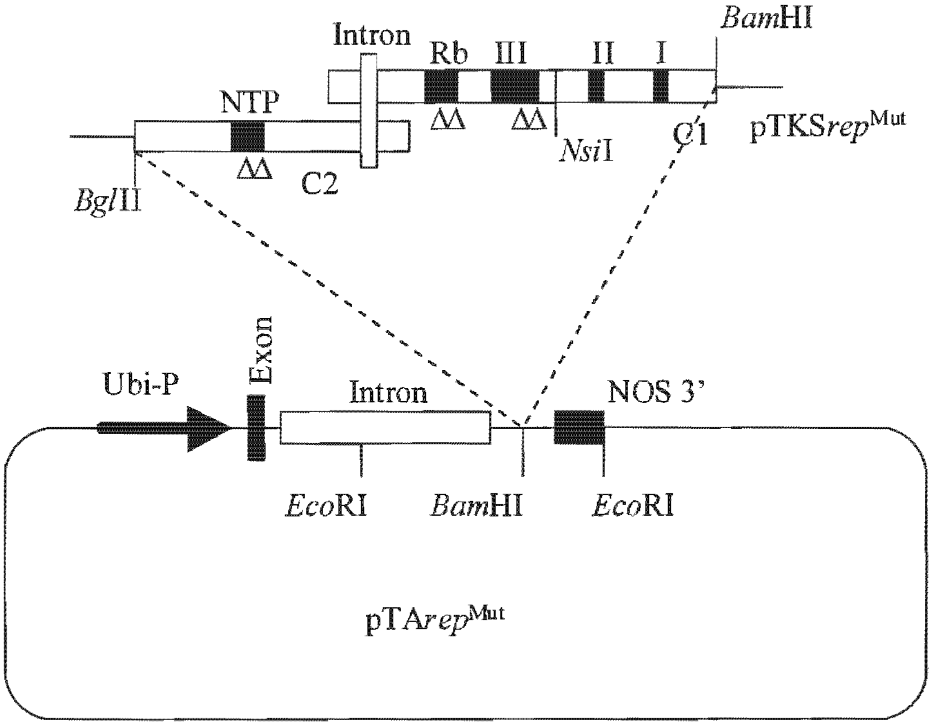


Figure 3.3. MSV-Kom Rep-encoding plant transformation constructs showing a representation of the plant vector pAHC17 (Carnejo *et al.*, 1993) and the derivatives pTA*rep*^{Mut}. Abbreviations: Ubi-P, Ubiquitin promoter; ^{Mut}, various combinations of mutations of the Rep domain (s), (▮) in the same plant vector (Table 3.1); NOS 3', nopaline synthase gene terminator; Δ, point mutation.

3.1.4 Transfection of BMS Suspension Cells by Particle Bombardment

For each transfection, BMS cells from four to five day cultures were filtered, thinly spread onto sterile Whatman No.1 filter paper and incubated on MS medium (Murashige and Schoog, 1962) supplemented with 12% sucrose for 12 hr prior to bombardment. Bombardment conditions were as described by Gordon-Kamm *et al.* (1991). A combination or individual plasmids were precipitated onto gold particles (1 μm; Bio-Rad) resuspended in 50% glycerol at 60 mg ml^{-1}. The precipitation was carried out stepwise, firstly by adding 1.2 μg of plasmid DNA while vortexing, followed by 50 μl of 2.5 mM $CaCl_2$ and lastly by 20 μl of 0.1 M spermidine. After washing twice with absolute ethanol, the precipitate was resuspended in 60 µl of absolute ethanol and divided for six shots. BMS cells were bombarded using the Helium Gene Delivery System (Bio-Rad) at 650 psi. Each bombardment represented an independent transfection. The replication assay was repeated three times.

3.1.5 Analysis of MSV DNA Replication in BMS Suspension Cells

MSV-Kom DNA replication was analysed using qualitative PCR (QPCR). This PCR was specific for replicating MSV-Kom and was not able to amplify parental plasmid DNA used for the transfection experiments. The degenerate primers MSVQPCR1 and MSVQPCR2 were designed such that they would anneal to amplify a target sequence which was interrupted by the parental plasmid in the vector: this would produce a 4.0-kb fragment, and the thermocycling conditions used in this experiment would not allow successful amplification of such a large fragment. The primers annealed to coordinates 210-191 and 1595-1613 of the MSV-Kom genome (Willment, 1999). They had the following nucleotide sequences: MSVQPCR1; 5'-CCAAA(A/T)(A/G/T)TCAGCTCCTCCG-3' and MSVQPCR2; 5'-TTGG(A/G/C)CCG(A/C)(A/G/C)GATGTA(G/C)A-3' where the brackets denote degenerate nucleotides. When the MSV-Kom genome was replicatively released, the primers would anneal to positions flanking a 1.3-kb target sequence. After 72 hr BMS cells were dried between sterile filter paper, and frozen in liquid nitrogen. DNA was isolated using the Nucleon Phytopure kit (Amersham International plc., Backinghamshire, England). One µg of DNA was amplified by PCR using primers MSVQPCR1 and MSVQPCR2. The DNA was amplified using the supplier's reaction buffer with the addition of 1.5 mM $MgCl_2$ and 0.25 U of *Taq* DNA polymerase. PCR was carried out using a Hybaid PCR Sprint or Perkin Elmer Thermocyler 9700. The PCR cycling conditions were: denaturing of DNA at 94°C for 2 min followed by 30 cycles at 94°C for 30 sec, 54°C for 30 sec, 72°C for 1 min and completion of the reactions at 72°C for 10 min.

Table 3.1. Plasmids used to assay for the replication efficiency of MSV-Kom containing mutated conserved domains of Rep.

Domains	**III$^+$**	**III$^-$**	**Rb$^+$**	**Rb$^-$**	**NTP$^+$**	**NTP$^-$**
↓→	$D^{99}\mathbf{Y}^{100}I^{101}$	$A^{99}\mathbf{Y}^{100}\mathbf{F}^{101}$	$N^{201}\mathbf{E}^{202}$	$L^{201}\mathbf{K}^{202}$	$Q^{248}N^{249}$	$P^{248}Y^{249}$
$X^{III+Rb+NTP+}$	+		+		+	
$X^{III-Rb-NTP-}$		+		+		+
$X^{III-Rb-NTP+}$		+		+	+	
$X^{III+Rb-NTP-}$	+			+		+
$X^{III-Rb+NTP-}$		+	+			+
$X^{III+Rb-NTP+}$	+			+	+	
$X^{III-Rb+NTP+}$		+	+		+	

→Amino acids targeted by PCR site-directed mutagenesis, ↓plasmids containing different mutations. The superscript numbers denote amino acid positions in the Rep protein of MSV-Kom and bold letters in the amino acid sequence denote a conserved residue. +, a conserved domain containing particular amino acids and $^-$ a mutated domain. III is the nicking and closing domain of MSV-wt Rep that has a characteristic VRDYIL**K** amino acid sequence, Rb is the retinoblastoma binding domain that has a conserved amino acid sequence **LLCNE** in MSV-Kom Rep and the NTP binding domain is a conserved $\mathbf{GX_4GKST}\mathrm{WARSX_7QNX_{13}NV}\mathbf{IDD}$ amino acid sequence were X denotes variable amino acids and the subscripts denote the numbers of the amino acids.

3.1.6 Transfection of BMS Suspension Cells to Analyse the *trans*-Replication Potential of MSV Rep Mutant Plasmids

The plant vectors in Figure 3.3 and the conserved domains described in Table 3.1, where X was substituted by pTA*rep*, were used to evaluate the efficiency of the mutated Rep to complement MSV carrying a defective Rep (pKEP177-*Pst*I) (Palmer, 1997) or Rep$^-$ MSV-Kom (p35-GUSLSIR; Figure 3.4) plasmids. The two plasmids were gifts from Drs Kenneth Palmer and Janet Willment, respectively (Palmer, 1997; Willment, 1999).

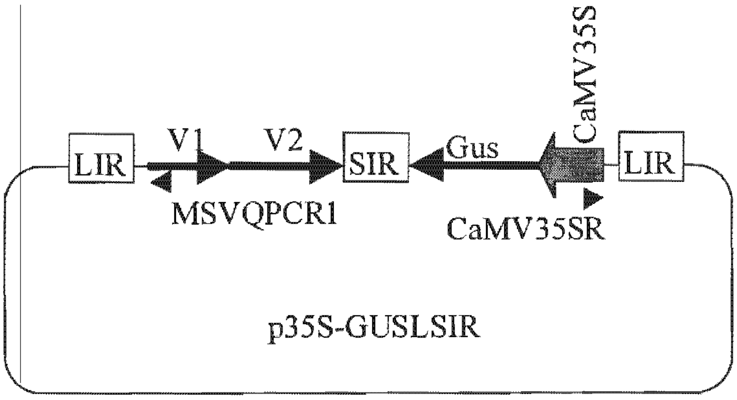


Figure 3.4. Schematic representation of construct p35S-GUSLSIR in which the Rep gene was replaced by CaMV35S-GUS (gift from Dr Janet Willment). *Trans*-replication was assayed using primers specific to the MP (movement) gene of MSV-Kom and the CaMV35S promoter with the sequence, 5'-CAACCACGTCTTCAAAGC-3' (from Genosystems Biotechnologies, France) at an annealing temperature of 56°C (the other conditions were the same as for QPCR). In the presence of functional Rep the MSV-Kom genome with the Rep substituted by CaMV35S-GUS is replicatively released by recombination of the LIRs. ▸, position of primers.

The construct p35-GUSLSIR was used to verify that the *trans*-replication of pKEP177-*Pst*I supported by pTA*rep*$^{\mathrm{Mut}}$ was not a result of fortuitous recombination that resulted in the reconstitution of the wild-type Rep gene in the plant vector. Transfection experiments were carried out as described elsewhere in this chapter except that two plasmids were co-bombarded into BMS cells, either pKEP177-*Pst*I or p35-GUSLSIR and the plant vector pTA*rep*$^{\mathrm{Mut}}$ (see Table 3.1 for the descriptions of Mut). QPCR was used to determine the ability of the mutant Rep plant vectors to complement the replication defective pKEP177-*Pst*I and p35-GUSLSIR constructs.

3.1.7 Agroinoculation of Maize Seedlings

Agrobacterium tumefaciens C58 containing the disarmed Ti plasmid pMP90 was transformed by the freeze-thaw method (Hoefgen and Willmitzer, 1988) with pTB*rep*$^{\mathrm{Mut}}$, pTB*rep*$^{\text{Wt}}$, pTB*rep*$^{\text{D99AYI}}$, pTB*rep*$^{\text{DY100FI}}$ and pTB*rep*$^{\text{DYI101F}}$ plasmids containing 1.1-mers of MSV-Kom (Figure 3.2). MSV-Kom genomes were introduced into susceptible Jubilee sweetcorn plants by agroinoculation as described by Grimsley *et al.*, (1987) and Martin *et al.*, (1999). Plants were maintained in an insect-free plant growth room with supplementary lighting to give a 16 hr photoperiod. The infected plants were monitored for symptoms on a daily basis.

3.2 RESULTS

3.2.1 PCR Analysis of Rep Mutants of MSV-Kom in BMS Suspension Cells

The ability of mutants to replicate was tested by bombarding BMS cells with mutant vectors in the series, pTK*rep*$^{\text{Mut}}$ (Figure 3.1). Qualitative (QPCR) was used to analyse DNA samples 72 hr after bombardment. This analysis is similar to that used by Hanson *et al.*, (1995), the major differences being the omission of *Dpn*I digestion of total DNA, which would remove input methylated plasmid DNA. I obtained the same result using samples digested with *Dpn*I prior to PCR amplification and samples that were not treated with *Dpn*I (result not shown). The results showed that the primers used were specific for replicatively released MSV-Kom DNA (Figure 3.5, panel A). Replication was undetectable in samples bombarded with pTK*rep*$^{\text{III-Rb-NTP-}}$, pTK*rep*$^{\text{III-Rb-NTP+}}$, pTK*rep*$^{\text{III-Rb+NTP-}}$, pTK*rep*$^{\text{III+Rb-NTP-}}$ and pTK*rep*$^{\text{III-Rb+NTP+}}$ showing that the mutated amino acid residues are essential for the replication of MSV-Kom. Unexpectedly, the construct pTK*rep*$^{\text{III+Rb-NTP+}}$, carrying mutations in the Rb-binding domain, replicated to comparable levels with the wild-type construct, pKom602 (Figure 3.5, panel (A) lanes 5 and 10). This mutant was analysed by restriction with *Bfr*I, a site included in the mutagenic primer: c▼tTaAG, where the lower case letters are the introduced nucleotide mismatches, to check whether the observed replication was due to contamination with wild-type MSV or to reversion of the mutant. The results shown in Figure 3.5, panel (B) (lanes 4 and 5) demonstrated that the site was apparently not altered. The restriction analysis produced the expected 1.128- and 0.178-kb fragments, indicating the presence of the engineered *Bfr*I restriction site in viral replicon DNA in BMS cells after 72 hr.

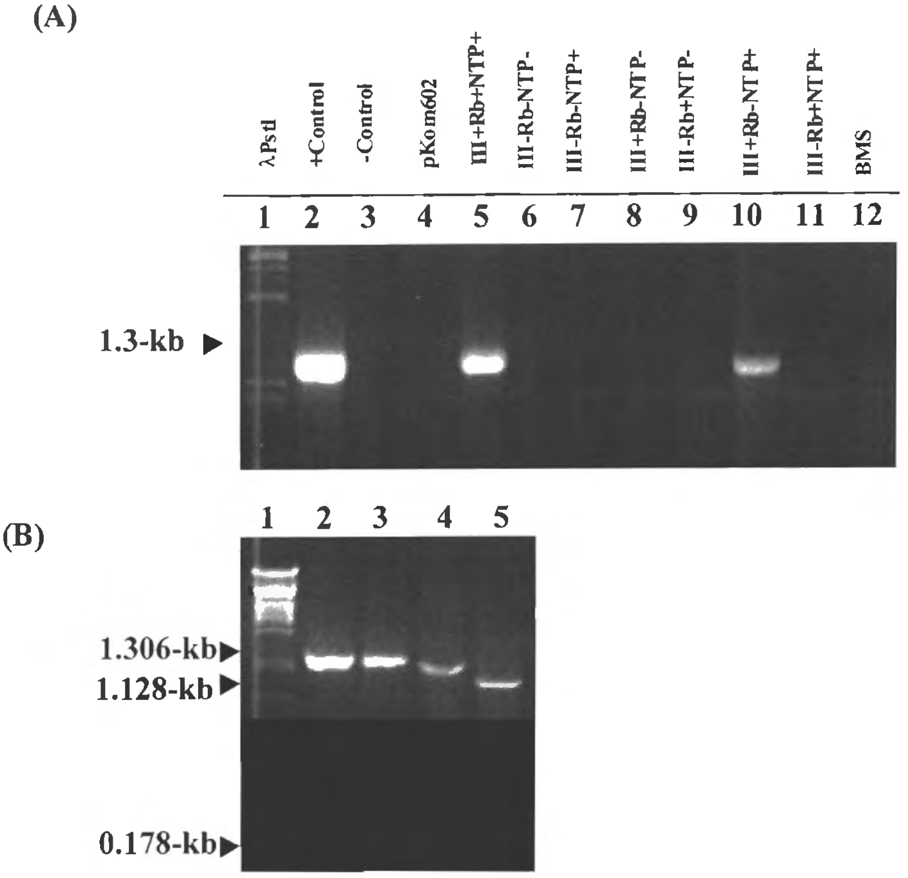


Figure 3.5. (A) QPCR analysis of the replication of MSV-Kom with the wild-type Rep gene fragment replaced by one carrying mutations (Lanes 6-11). One tenth of the total PCR reaction volume was loaded in each lane. Ten ng of total DNA from MSV-Kom infected maize was used as a + ve control (Lane 2). Lanes: 1, λ*Pst*I DNA marker; 3, water in place of DNA; 4, 10 ng of plasmid pKom602; 5, total DNA from BMS bombarded with pKom602 (=III^{+}Rb^{+}NTP^{+}); 6-11, constructs carrying different mutations to the conserved Rep protein domains; 12, mock bombarded BMS cells. $^{+}$ wild-type domain and $^{-}$ mutated domain as described in Table 3.1. (B) Lanes: 1, λ*Pst*I DNA marker; 2, undigested III^{+}Rb^{+}NTP^{+} PCR product; 3, as for 2, digested with *Bfr*I; 4, undigested III^{+}Rb^{-}NTP^{+} PCR product and 5, as for 4, digested with *Bfr*I to produce two fragments of 1.128-kb and 0.178-kb in size.

3.2.2 Analysis of the *trans*-Replication of a Defective Rep MSV-Kom by pTA*rep*$^{\text{Mut}}$ Constructs

The ability of the mutants to *trans*-replicate pKEP177-*Pst*I, a defective Rep MSV-Kom construct, was evaluated by co-bombarding BMS cells with pTA*rep*$^{\text{Mut}}$ (Figure 3.3) and pKEP177-*Pst*I. QPCR was used to amplify replicatively released MSV-Kom from total DNA isolated 72 hr after bombardment. The expected 1.3-kb fragments were obtained from constructs pTA*rep*$^{\mathrm{III+Rb+NTP+}}$ and pTA*rep*$^{\mathrm{III+Rb\text{-}NTP+}}$. These results showed that the wild-type Rep construct supported *trans*-replication and the constructs were expressing the gene product of interest. The construct containing the mutations N^{201}→L and E^{202}→K in the L^{198}LCNE^{202} Rb-binding domain unexpectedly supported *trans*-replication. Replication was not detected in any of the samples co-bombarded with the other mutants (Figure 3.6). The strategy used and the results obtained are similar to those obtained for the bipartite geminivirus TGMV Rep or Rep mutants, homologues of MSV-Kom Rep, where complementation experiments rather than western blots were used to ensure that the desired gene product was expressed (Sunter *et al.*, 1993).

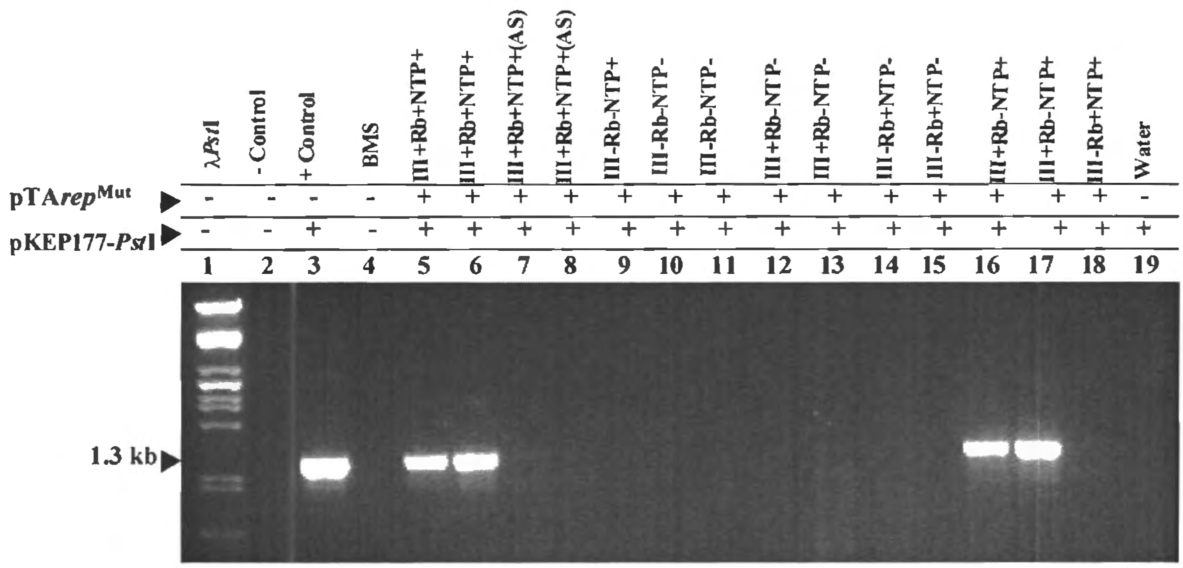


Figure 3.6. *Trans*-replication of pKEP177-*Pst*I by the Rep mutants introduced in the conserved domains of the Rep protein. QPCR fragments were amplified from replicative viral DNA and separated on 1.2% ethidium bromide-stained agarose gel. Lanes: 1, λ*Pst*I marker; 2, water as a template (negative control); 3, 10 ng of MSV infected total DNA (positive control); 4, BMS mock bombarded with gold particles without DNA (negative control); 5-6, wild-type Rep sense construct and pKEP177-*Pst*I; 7-8, wild-type Rep antisense construct and pKEP177-*Pst*I; 9-18, mutant plasmids and pKEP177-*Pst*I; 19, pKEP177-*Pst*I and water.

To verify that the *trans*-replication of pKEP177-*Pst*I was not the result of a productive recombination between the Rep genes of itself and the trans-activator plasmid, I transfected BMS suspension cells with p35-GUSLSIR (Figure 3.4) and pTA*rep*$^{\mathrm{Mut}}$ as previously described. The former contains CaMV35S-GUS in place of the Rep gene between the LIR and SIR in the MSV-Kom genome. It carries an additional LIR upstream of V1 and V2 to enable recombinational or replicative release and efficient replication (see Stenger *et al.*, 1991) of the MSV-CaMV35S-GUS genome in the presence of functional Rep. This was detected by QPCR using primers MSVQPCR1 and CaMV35SR (Figure 3.7). The results confirmed those obtained using pKEP177-*Pst*I (Figure 3.6). The expected 0.806-kb fragments were obtained from samples bombarded with the Rb⁻ mutants and the wild-type Rep (Figure 3.7, lanes 12, 13 and 15), while the other mutant constructs did not support the *trans*-replication of the p35-GUSLSIR construct. There are presumably non-specific bands of more than 1.3-kb in lanes 4, and 6-14, probably due to alternative annealing sites in the plasmid p35-GUSLSIR. The bands could not have been due to primers annealing to the genomic sequences due to their absence in, lane 2 which carries BMS DNA alone. There are very faint specific bands of 0.806-kb in size lanes 8-11 that could be a result of mutant Rep having some residual functional activity.

These results demonstrate that the *trans*-replication of defective Rep MSV-Kom was not due to a rare event of recombination that resulted in the reconstitution of a functional Rep protein.

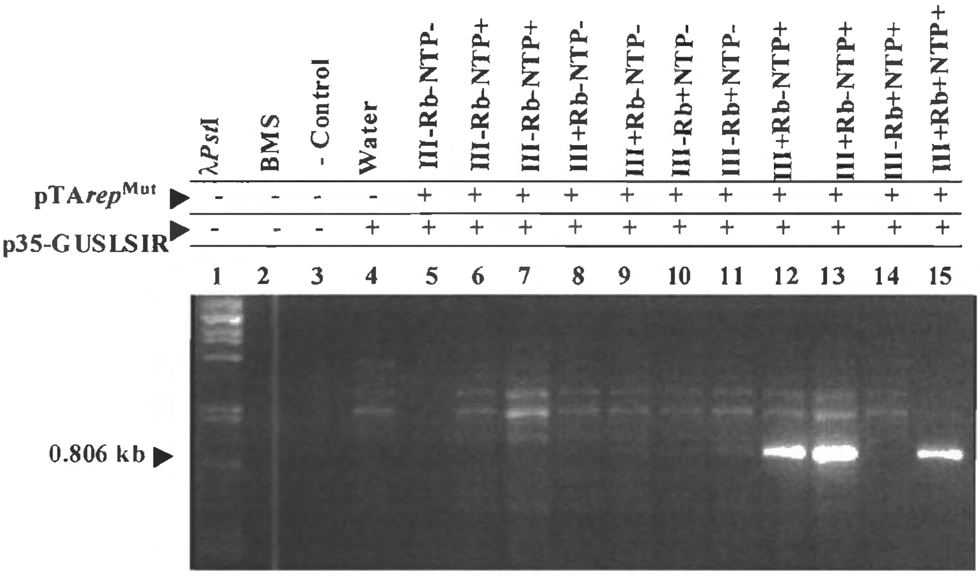


Figure 3.7. *Trans*-replication of a p35-GUSLSIR plasmid by Rep mutant plant vectors. One µg of total DNA from bombarded samples was amplified using QPCR. Primers MSVQPCR1 and CaMV35S, specific to the p35-GUSLSIR, construct were used. Lanes: 1, λ*Pst*I marker DNA; 2, BMS mock bombarded with water instead of DNA; 3, water (negative control); 4, p35-GUSLSIR construct only; 5-14, mutant constructs and p35-GUSLSIR; 15, wild-type Rep and p35-GUSLSIR plasmid.

3.2.3 Analysis of the Infectivity of MSV-Kom Rep Mutant Constructs *in planta* using *Agrobacterium tumefaciens* C58 for Agroinfection

To evaluate the infectivity and symptom development of the various MSV-Kom Rep mutant constructs, susceptible Jubilee sweetcorn maize seedlings were agroinfected with the constructs shown in Figure 3.2. Two sets of agroinoculation experiments were carried out. The first set involved constructs carrying double mutations within a single domain, and different permutations of the mutant domains. The second set consisted of the individual amino acid mutations within the nicking-closing motif III. In the first set twenty-eight maize seedlings per construct were agroinfected and scored for symptoms two weeks post-infection. Leaf samples from ten plants per construct were used for membrane squash-blot PCR analysis. The results are shown in Table 3.2.

Table 3.2. Analysis of the infectivity and symptom development of MSV-Kom Rep mutant *in planta*.

Vector	No. of plants with symptoms	Membrane blot PCR analysis[a]
Control	0	0/10
pTB*rep*$^{\mathrm{III+Rb+NTP+}}$	28	10/10
pTB*rep*$^{\mathrm{III-Rb-NTP-}}$	0	0/10
pTB*rep*$^{\mathrm{III-Rb-NTP+}}$	0	0/10
pTB*rep*$^{\mathrm{III-Rb+NTP-}}$	0	0/10
pTB*rep*$^{\mathrm{III+Rb-NTP-}}$	0	0/10
pTB*rep*$^{\mathrm{III+Rb-NTP+}}$	20	10/10
pTB*rep*$^{\mathrm{III-Rb+NTP+}}$	0	0/10

[a] Squash membrane blots from selected plants were subjected to PCR amplification using primers MSVQPCR1 and MSVQPCR2. Control, *Agrobacterium* without binary vector.

The construct containing the mutation to the Rb-binding domain showed 71% infectivity with symptoms appearing two weeks after agroinfection. The symptoms were attenuated in these plants compared with those agroinfected with wild-type MSV. The symptom severity was quantified using a digital-imaging system (Martin and Rybicki, 1998; Figure 3.8). The differences in symptom severity are clearly marked when samples 1 and 2 in Figure 3.8 are compared. The agroinfectious construct carrying the Rb$^-$ mutant infected a smaller number of plants relative to the wild type (Table 3.2), and produced attenuated symptoms (Figure 3.8).

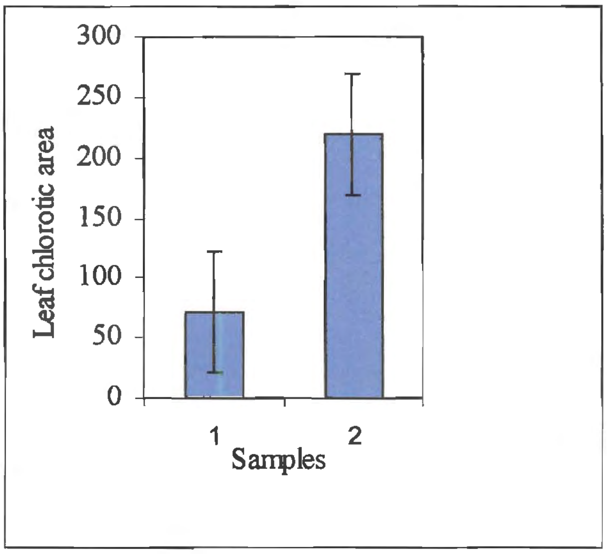


Figure 3.8. Estimation of symptom severity using digital image analysis of the third leaf of all the plants showing symptoms. Samples: 1, the symptoms due to the agroinfectious construct, pTB*rep*$^{III+Rb-NTP+}$ carrying the Rb-binding mutation and 2, wild-type agroinfectious construct, pTB*rep*$^{III+Rb+NTP+}$. The error bars indicate variation of the mean of individual leaf samples of the tested plant population.

The second set of experiments evaluated the importance of individual amino acids constituting the nicking-closing domain in motif III of MSV-Kom Rep or RepA using agroinfection (Table 3.3). A total of 36 plants per construct were agroinfected and the experiment was repeated three times. None of the plants tested using the mutant MSV-Kom plasmids developed any symptoms over a period of 1-2 months. Control plants inoculated with wild type MSV-Kom were included in every experiment. The control plants inoculated with wild type MSV-Kom developed severe symptoms 1 to 2 weeks post-infection. The results demonstrated that the nicking-closing motif of MSV-Kom does not tolerate any mutations (Table 3.3). In addition the variant amino acids flanking the conserved tyrosine residue are important for the initiation and / or termination of replication. As no assays were done to detect replication of MSV-Kom, the absence of symptoms does not necessarily mean the complete absence of replication. Rather the absence of symptoms could be due to residual replication of MSV-Kom that is below the threshold level for the development of symptoms (Wartig *et al.*, 1997). However, I can conclude that the mutations introduced effectively abolished the infectivity of MSV-Kom.

Table 3.3. The infectivity of mutants of the nicking-closing domain of motif III of MSV-Kom in susceptible Jubilee sweetcorn maize plants.

Mutant ORF	**Inoculum**	**Symptomatic plants**
Wt	pTB*rep*$^{\text{DYI}}$	108[a]\108
C1	pTB*rep*$^{\text{D99AYI}}$	0\108
C1	pTB*rep*$^{\text{DY100FI}}$	0\108
C1	pTB*rep*$^{\text{DYI101F}}$	0\108
C1	pTB*rep*$^{\text{D99AYI101F}}$	0\108

[a]Severe MSV-Kom symptoms. 108 is the total number of plants inoculated for the three experiments.

3.3 DISCUSSION

Mastreviruses have reduced coding capacity and depend on the host machinery for all replication and transcription functions, except for the replication initiator protein (s). In order to utilise the host replication machinery the viruses must be capable of recruiting and linking the replication factors to the viral cycle. To investigate the importance of both conserved and variant amino acids within the targeted functional domains of MSV-Kom Rep or RepA I carried out mutational analysis both with rapidly dividing maize tissue-cultured cells, and *in planta*.

The primary event upon MSV invasion of the host cell is the synthesis of a second strand complementary to the virion DNA, using an 80-bp primer included in the virion (Donson *et al*., 1984). The replicative form (RF)-DNA produced by priming of a site within the SIR is used as a template for MSV-Kom replication. Geminiviruses replicate by a rolling circle (RC) mechanism identical to that used by some plasmids and phages in bacteria (Kornberg and Baker, 1992; del Solar *et al*., 1993) and parvoviruses in animal cells (Snyder *et al*., 1990; Ni *et al*., 1994). In geminiviruses, the amount of the Rep protein is rate-limiting, and it is absolutely required for virus replication (Frischmuth and Stanley, 1991). To initiate replication, the Rep binds to the origin of replication and introduces a nick at the conserved nonanucleotide TAATATT$^{7}\downarrow^{8}$AC in the (+) strand of the dsDNA to generate a free 3'-OH end that is used to prime DNA synthesis. The synthesis is terminated on completion of a full round of replication by a second cleavage and ligation to release a monomeric genome and a new double stranded DNA copy.

The *cis*-elements required for DNA binding and cleavage have been demonstrated for the AC1\C1 of begomo- and curtoviruses (Fontes *et al*., 1994; Laufs *et al*., 1995; Orozco *et al*., 1997), RepD of plasmid pT181, the gene A product of ΦX174, Tra protein of RP4 and Mob protein of plasmid RSF1010 (Panseguru *et al*., 1990; Thomas *et al*., 1990; Rasooly and Novick, 1993; Scherzinger *et al*., 1992). In MSV-Kom Rep or RepA motif III has an amino acid sequence RDYILK, where the **Y** residue is conserved in Rep proteins of all geminiviruses and RCR replicons. The functional importance of the conserved **Y** residue has been demonstrated in the AC1\C1 of TGMV, TYLCV, BGMV and ACMV (Orozco and Hanley-Bowdoin, 1998; Laufs *et al.*, 1995; Hoogstraten *et al.*, 1996; Stanley, 1995). In this work, instead of mutating the conserved **Y** only, I also targeted the flanking amino acids using an approach similar to that reported by Hoogstraten *et al.*, (1996). The acidic or γ-carboxylate aspartate and lysine contribute to the cleavage activity of the conserved **Y** of RCR initiator proteins that act in a topoisomerase type I-like manner (Noirot-Gros *et al.*, 1994; Nuesch *et al.*, 1995; Berger *et al.*, 1998). MSV-Kom Rep or RepA residue $\mathbf{Y}^{100}$ is flanked by D^{99} (a γ-carboxylate amino acid) and I^{101}.

I evaluated the ability of the mutant plasmid pTK*rep*^{D99AYI101F} to replicate in BMS cells derived from the base of meristematic leaf tissue. The Chou-Fasman predicted secondary structure showed conservation in the nicking-closing domain. The plasmid carrying the mutations $D^{99}A$ and $I^{101}F$ did not replicate in BMS cells indicating that these amino acids were also essential for the replication of MSV-Kom; however, I could not tell if either or both were essential. The contribution of the individual amino acids in the nicking-closing domain of motif III was evaluated by agroinfection experiments. The mutants $D^{99}A$, $\mathbf{Y}^{100}F$ and $I^{101}F$ were not agroinfectious demonstrating the importance of the individual amino acids in MSV-Kom infectivity or symptom development. It has been reported in TGMV Rep mutational analysis that the change $\mathbf{Y}^{103}F$ was conservative because the mutant protein bound to DNA to comparable levels with the wild type protein (Orozco and Hanley-Bowdoin, 1998). The Chou-Fasman predicted secondary structure did not show any remarkable changes. The mutant $\mathbf{Y}^{100}F$ probably binds to the origin of MSV-Kom but fails to nick the + strand of DNA. The aromatic ring of the conserved **Y** contributes to binding while the -OH group is required for DNA cleavage.

Changing **Y**→F does not alter the aromatic ring, instead the -OH group is replaced by -H. Experiments with TYLCV Rep and BGMV Rep demonstrated that $\mathbf{Y}^{103}$ is necessary for viral replication (Laufs *et al.*, 1995; Hoogstraten *et al.*, 1996).

MSV-Kom contains a highly charged D^{99} upstream of the active $\mathbf{Y}^{100}$. The $D^{99}A$ mutation resulted in **AYI** identical to that of BGMV Rep. This mutation was not agroinfectious. The Chou-Fasman predicted secondary structure showed remarkable conformational changes. These changes probably impaired binding of Rep to the origin and cleavage. These results are consistent with those obtained for TGMV Rep mutants containing alanine substitutions for the three aspartic acids ($D^{105}A$, $D^{107}A$ and $D^{109}A$) that failed to support replication (Orozco and Hanley-Bowdoin, 1998). The mutant $I^{101}F$ failed to agroinfect and the Chou-Fasman predicted secondary structure showed an apparent increase in hydrophilicity. This increase probably reduces the competitiveness of the mutant Rep protein to bind to the origin of replication. The other probable reason is that the amino acid mutated is close to the active site, making it possible for any minimal changes to abolish MSV-Kom replication. The effect of these mutations in inhibiting MSV-Kom replication can not be inferred without carrying out further experiments to establish competition.

The second domain that was targeted and evaluated using the BMS cells was the Rb-binding domain. As the virus depends on the host for replication, there must be a link between the virus and the host replication machinery. The oncoproteins of animal DNA tumour viruses share the **LxCxE** Rb-binding motif with all of the characterized members of the genus *Mastrevirus,* except for SSV that has an imperfect **LxxxE** motif (Hughes *et al.*, 1993). The existence of a retinoblastoma analogue protein in plants, including maize, has been demonstrated (Xie *et al.*, 1996; Grafi *et al.*, 1996; Ach *et al.*, 1997; Huntley *et al.*, 1998). The plant mastreviruses are likely to operate in a similar manner to mammalian DNA tumor viruses by targeting and inactivating the Rb, which if undisturbed induces the growth phase arrest and differentiation. Viral oncoproteins bind to Rb via the **LxCxE** motif resulting in Rb inactivation, which mimics that of cyclins and Rb. The latter interaction is also via the **LxCxE** but results in inactivation due to phosphorylation of the Rb mediated by cyclin activated kinases known as cyclin dependent kinases (cdks). Mutants of Ad E1A, SV40 large T antigen and human papilloma E7 viral oncoproteins that have lost their ability to bind Rb and other Rb-related proteins, exhibit significantly reduced transforming potential (Moran *et al.*, 1986; Lille *et al.*, 1987; Cherington *et al.*, 1988). The **LxCxE** motif is also found in *Vir*B4 of *Agrobacterium tumefaciens* (Hooykas and Berijersbergen, 1994) and histone deacetylase (HADC1: Ferreira *et al.*, 1998; Brehm *et al.*, 1998; Luo *et al.*, 1998; Magnaghi-Jaulin *et al.*, 1998). *Vir*B4 is involved in the interaction of the crown gall-causing bacterium with the host plant, while HADC1 is involved in chromatin modelling. Acetylated histones are characteristic of actively transcribed genes while inactive or repressed genes exhibit little chromatin acetylation (for review, see Struhl, 1998). Acetylation of specific lysine residues in the N-termini of histones is believed to weaken their interaction with DNA.

I mutated the **LLCNE** to **LLCLK**. The predicted Chou-Fasman plotstructure was the same as that of mutations RA^{99}**Y**F^{101}L although different amino acids were mutated (see Chapter 2). Although an intact Rb-binding motif is required for the efficient replication of WDV (Xie *et al.*, 1996), the Rb-mutant plasmid, pTK*rep*$^{\text{III+Rb-NTP+}}$, replicated in BMS to levels comparable with the wild type. BMS cells derived from the meristematic region of the leaf base are normal mitotic cells unlike those from the endosperm (Grafi *et al.*, 1996) that double their DNA content by endoreduplication instead of mitosis. Rapidly dividing BMS cells have very low levels of Rb while differentiated cells have high levels of Rb. In this experiment, the mutant Rep is likely to interact with the MSV-Kom origin of replication making available nicked MSV that can readily replicate.

The mutant plasmid, pTK*rep*$^{\text{III+Rb-NTP+}}$, that replicated in BMS was analysed by restriction with *Bfr*I to determine whether the mutations introduced were maintained or not. The results showed that the engineered *Bfr*I site (Figure 3.5 panel B) was not changed after 72 hr in BMS.

The third motif targeted was the NTP-binding domain commonly found in kinases like CDC48 and helicases (Gorbalenya *et al.*, 1990, Feiler *et al.*, 1995). The helicase-like motif exhibits DNA-dependent ATPase activity and is involved in unwinding dsDNA templates making easy access to the replication fork. The NTP and helicase domain of MSV-Kom is found in the Rep and not RepA and is represented by amino acids $\mathbf{G}\text{X}_4\mathbf{GKST}\text{WARSX}_7\text{Q}^{248}\text{N}^{249}\text{X}_{13}\text{NIV}\mathbf{DD}$ where **G**, **K**, **S**, **T** and **D** are conserved in geminiviruses. Instead of targeting the conserved amino acids I mutated the variant $\text{Q}^{248}\rightarrow\text{P}$ and $\text{N}^{249}\rightarrow\text{Y}$ that are close to the transactivation domain between amino acids 252 and 270. It is interesting to note that the NTP\ATPase-helicase and the transactivation domain overlap (Horvath *et al.*, 1998) and changes to the amino acids close to or within the overlap will probably affect both functions. The Chou-Fasman (1978) predicted secondary structure of mutants to this domain showed extensive changes that may impact on the overall function of the Rep protein. Hanson *et al.*, (1995) demonstrated the importance of the conserved amino acids within the putative NTP-binding domain in the Rep protein of BGMV on viral replication. In TYLCV Rep, a change of the K^{227} abolished both replication of the virus and the ATPase activity of the Rep *in vitro* (Desbiez *et al.*, 1995).

In this study I was not able to introduce the $Q^{248} \rightarrow P$ and $N^{249} \rightarrow Y$ in an individual plasmid as a single domain mutant but I introduced the mutants in combination with others. Mutants of the non-conserved amino acids within the NTP\ATPase-helicase domain are important for MSV-Kom replication as can be deduced from the failure of construct pTK*rep*$^{III+Rb-NTP-}$ to replicate while construct pTK*rep*$^{III+Rb-NTP+}$ replicated. This is in contrast to the finding that mutations to variant amino acids within the NTP\ATPase-helicase binding motif of BGMV Rep had no effect on viral replication (Hanson *et al.*, 1995). The mutant constructs pTK*rep*$^{III-Rb-NTP-}$, pTK*rep*$^{III-Rb+NTP-}$, and pTK*rep*$^{III+Rb-NTP-}$ did not replicate to detectable levels indicating that the amino acid changes or a combination thereof with mutations in other domains are critical for MSV-Kom replication.

The non-replicating constructs have possible use as sources of MSV-Kom resistance in transgenic plants. I investigated their potential by using them to *trans*-replicate defective or Rep- MSV-Kom constructs, similar to what was reported for WDV and ACMV (Timmermans *et al.*, 1992; Etessami *et al.*, 1991). I used two different replication-incompetent MSV-Kom constructs to account for possible fortuitous recombination that might restore Rep or RepA function. This experiment was also used to demonstrate that the protein of interest was expressed without using western blot analysis. The plant vectors (Figure 3.3) were co-bombarded separately with the defective

MSV-Kom Rep or Rep- constructs (Figure 3.4). The construct pTA*rep*$^{\mathrm{III+Rb+NTP+}}$ was used as a positive control and it complemented the replication of the two defective constructs. The ability of this construct to support replication was indicative of the expression of the protein of interest. The plasmid pTA*rep*$^{\mathrm{III+Rb-NTP+}}$ also supported replication. Although this was unexpected, it showed that the mutant protein produced served no overall negative regulatory function in the replication of MSV-Kom in BMS cells (Figures 3.6 and 3.7). The fact that the tests were done in rapidly dividing cells makes the requirement for an intact Rb-binding motif less relevant.

Plasmids pTA*rep*$^{\mathrm{III+Rb+NTP+(AS)}}$, $^{\mathrm{III-Rb-NTP-}}$, $^{\mathrm{III-Rb-NTP+}}$, $^{\mathrm{III+Rb-NTP-}}$, $^{\mathrm{III-Rb+NTP-}}$ and $^{\mathrm{III-Rb+NTP+}}$ failed to *trans*-replicate the defective MSV-Kom constructs, pointing to the potential use of these constructs in the development of MSV-Kom resistance. The Chou-Fasman prediction of the secondary structures of the different mutants and combinations thereof demonstrated extensive changes in the NTP\ATPase-helicase region (Figure 2.3, Chapter 2).

The infectivity and symptom development of the MSV-Kom Rep mutant constructs was evaluated by agroinfecting constructs into susceptible Jubiliee sweetcorn seedlings (Figure 3.2). Wild type and Rb-mutant constructs displayed marked differences in the number of plants infected and the severity of symptoms. PCR analysis of infected material showed the presence of replicating DNA (Table 3.2). The agroinfectious Rb-mutant (Table 3.2 and Figure 3.8) clearly demonstrated the attenuation of symptoms when compared to the wild type. These results compare well with those obtained by Liu *et al.*, (1999) who reported a severe reduction in the severity of symptoms due to the mutation **E**→Q. The mutants I introduced are different from those by Liu and co-workers, and no binding assays were carried out. The effect of the mutations N^{201}→L and E^{202}→K on leafhopper transmissibility of MSV-Kom, and mutant reversion or changes after repeated cycles of transmission are under investigation. The other mutants were not agroinfectious, confirming the result obtained in BMS cells.

The constructs that did not replicate in BMS, failed to *trans*-replicate defective MSV and were not agroinfectious, were selected for further analysis with the ultimate goal of developing maize resistant to MSV. The system I developed for testing genetic constructs is efficient in supplying necessary information on the replication of MSV-Kom before embarking on the development of transgenic plants.

CHAPTER FOUR

CONTENTS

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CHAPTER FOUR

The *Trans*-Dominant Effect of Rep Mutants on the Replication of MSV in Black Mexican Sweetcorn Suspension Cells

ABSTRACT

To develop MSV-Kom Rep or RepA mutants that exert a dominant inhibitory effect on MSV-Kom replication I targeted the domains exhibiting nicking-closing, retinoblastoma binding and NTP\ATPase-helicase activities. I tested several plasmids carrying single, double or triple domain mutations for their ability to support or inhibit viral DNA replication in BMS cells. Single-domain mutants of the nicking-closing motif inhibited replication of MSV-Kom to undetectable levels in BMS suspension cells. Double-domain mutants of the nicking-closing and Rb motifs as well as the NTP\ATPase-helicase and Rb motifs were unable to support MSV replication. The double-domain mutant of the nicking-closing and NTP\ATPase-helicase motifs supported limited replication of MSV. These results suggest that the inhibition of MSV replication is probably due to competition between the mutant and wild type forms of Rep or RepA for binding to the viral origin of replication.

4.0 INTRODUCTION

There are a few examples where conventional breeding methods are effective in controlling diseases caused by geminiviruses (Frischmuth and Stanley, 1993). An effective resistance strategy entails an in-depth understanding of the molecular mechanisms underlying geminiviruses replication and movement. Viral genes and the proteins involved in replication and movement from cell-to-cell (short distance) have been studied but a lot more needs to be unravelled. Several strategies for viral resistance utilising the CP, Rep and MP of geminiviruses have been demonstrated in different transgenic crops (Kunik *et al.*, 1994; Noris *et al.*, 1996; Hong and Stanley, 1996; Brunneti *et al.*, 1997; von Armin and Stanley, 1992; Duan *et al.*, 1997). In addition to the protein-mediated form of resistance, other non-viral protein strategies such as defective interfering (DI) DNA and antisense mRNA have been used to produce transgenic plants resistant to diseases caused by geminiviruses (Stanley *et al.*, 1990; Frischmuth and Stanley, 1991; Day *et al.*, 1991; Frischmuth *et al.*, 1997).

In most of the cases where the geminivirus Rep protein was used, part of the C-terminus was truncated and the Rep gene encoded a limited portion of the N-terminus (TYLCV C1: Noris *et al.*, 1996; ACMV AC1: Hong and Stanley, 1996; TYLCV C1: Brunetti *et al.*, 1997). Two interesting examples of modified Rep were demonstrated in transgenic tobacco plants, resistant to geminiviruses, *Tomato mottle virus* (TMoV) and *Cabbage leaf curl virus* (CabLCV). The transgenic tobacco plants expressed a truncated BC1 or mutant BC1 (with an insert of 26 amino acids in the BC1 ORF from an unidentified source of origin) derived from TMoV (Duan *et al.*, 1997). The Rep or movement proteins were used as dsyfunctional proteins (truncated or with an insert) implicating a "dominant negative interference". The criterion for a gene to be a “dominant negative mutant” is determined by its ability to interfere with the function (s) of a wild type gene and over-expression of the target gene may sometimes overcome the negative dominance (Amaya *et al.*, 1991).

The use of dominant negative mutants, as described elsewhere in this thesis, is an attractive and multi-stage strategy to confer virus resistance (for review, see Herskowitz, 1987). This strategy is well illustrated in the *trans*-dominant inhibition of *Herpes simplex virus* (HSV) in transgenic mice expressing the mutant allele (X25) of an essential regulatory protein ICP4 (Smith and DeLuca, 1992). The ICP4, like the Rep protein of geminiviruses, is involved in the transcription of late genes (DeLuca and Schaffer, 1988), is phosphorylated, localised to the nucleus (Courtney and Benyesh-Melnick, 1974), exists as a homodimer (Shepard and DeLuca, 1989) and binds DNA with some specificity (Muller, 1987; Faber and Wilcox, 1986). I propose that the use of a full length mutant Rep or RepA would result in "multi-stage" dominant negative interferences. I report the *trans*-dominant inhibitory effect of various mutants of the full-length MSV-Kom Rep or RepA proteins on the replication of MSV-Kom using a transient BMS expression system.

4.1 MATERIALS AND METHODS

4.1.1 Construction of Plant Vectors

The plant vectors designated pTA*rep*$^{\mathrm{Mut}}$ constructed as described in Figure 3.3 were used to evaluate the *trans*-dominant effect of various mutations to the conserved functional domains of MSV-Kom Rep protein.

4.1.2 Transfection of BMS Suspension Cells by Particle Bombardment

The transfection experiments were carried out essentially as described in the Materials and Methods of Chapter 3, but with the plant vectors pTA*rep*$^{\mathrm{Mut}}$ being co-bombarded with an MSV-Kom vector, pKom602. This was to assay the inhibitory effect of expression of defective Rep on MSV replication. The BMS cells used were maintained and treated as previously described, and the experiments were repeated three times.

4.1.3 PCR Analysis of MSV Replication in BMS Suspension Cells

Total DNA isolated using the Phytopure kit was amplified by the MSV-Kom replication specific PCR outlined in Figure 4.1, panel 2. The DNA samples analysed by qualitative PCR (QPCR) in Figure 4.2 were subjected to quantitative PCR as shown in Figure 4.1, panel 3. Thermocycling conditions previously described for QPCR in Chapter 3 were used but the concentration of the internal standard, pKEP177-*Pst*I, ranged from 1×10^{-10} to 100 ng as shown in Figure 4.3 (A).

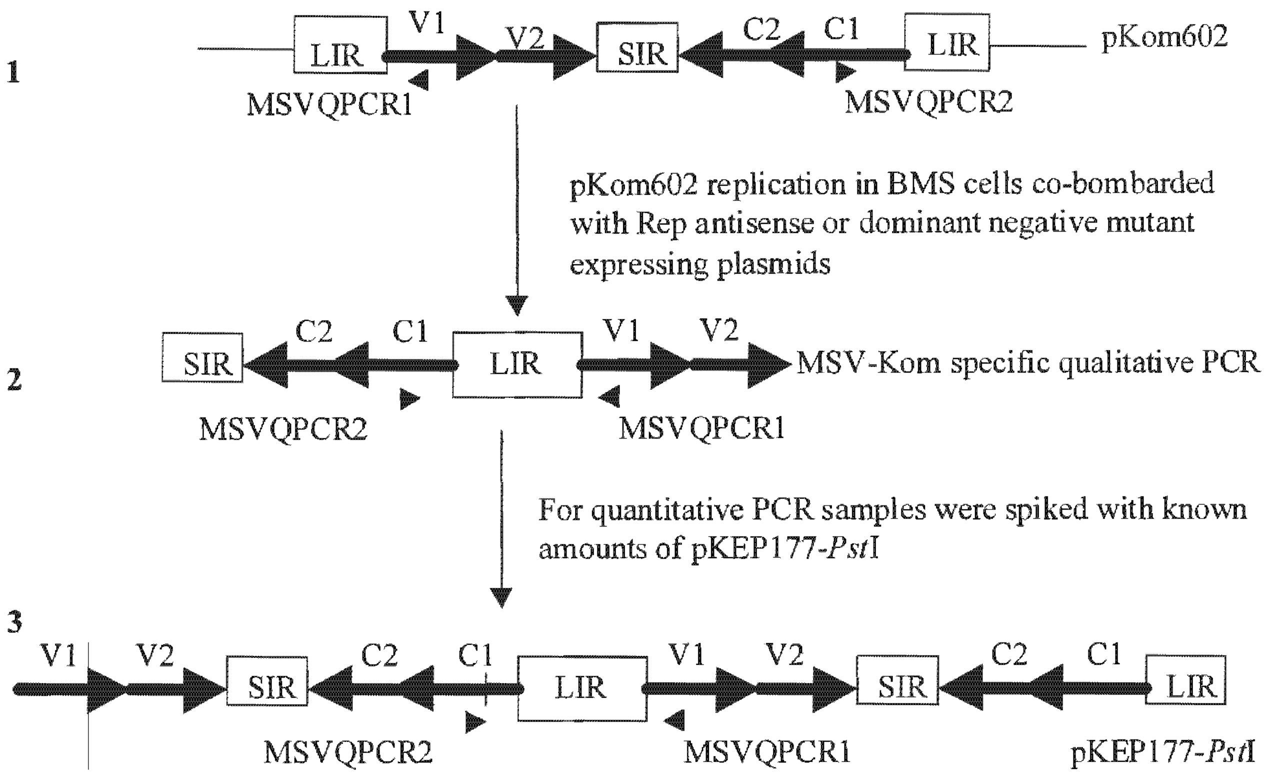

Figure 4.1. The qualitative and quantitative PCR assays for MSV replication by selective PCR amplification of replicating MSV-Kom. 1, non-replicating vector (the MSV-Kom 1.1-mer in pUC19 can not be amplified under the conditions used); 2, replicating MSV-Kom is amplified using qualitative PCR (results shown in Figure 4.2); 3, Rep RSQ-PCR (= replication specific) quantitative PCR with pKEP177-*Pst*I (engineered site) as an internal standard and total DNA from bombarded samples as competitor (results shown in Figure 4.3). ◀ ▶, arrow points in the direction of priming. |, PCR-added *Pst*I restriction site.

4.1.4 Replication Assay using Southern Blot Analysis

Ten μg of total DNA from bombarded samples was fractionated by electrophoresis through 1.0% ethidium bromide-stained agarose gel in TBE buffer and transferred onto Hybond N+ membrane (Amersham International) using the alkaline-NaCl method. Membrane bound total DNA was hybridised to the MSV-Kom Rep gene which was labelled with $[\alpha]$-^{32}P-dCTP using a Random Primed DNA labelling kit (Boehringer Mannheim Biochemicals, Indianapolis, IN, USA). Pre-hybridisation and hybridisation were carried out at 60°C in 6 X SSC, 5 X Denhardt's solution, 20 mM Tris-HCl pH 7.6, 0.8% SDS, 100 μg/ml denatured salmon sperm DNA. After overnight hybridisation membranes were briefly washed in 2 X SSPE + 0.1% SDS, and twice for 15 min with 0.1 X SSPE + 0.1% SDS at 60°C. Filters were exposed at –70°C to X-ray film with an intensifying screen. Films were developed after 4 days and the bands subjected to densitometry by digital image analysis (Hoefer Scientific Instruments).

4.2 RESULTS

4.2.1 Qualitative PCR Analysis of Inhibition of MSV Replication using *trans*-Dominant Rep Plant Vectors

BMS suspension cells were co-bombarded with MSV-Kom and mutant Rep constructs to assess the *trans*-dominant effects of the mutations on MSV-Kom replication (Figure 4.3). DNA from different samples was assayed using QPCR (Figure 4.2). The constructs pTA*rep*$^{\text{III-Rb-NTP-}}$, pTA*rep*$^{\text{III-Rb-NTP+}}$, pTA*rep*$^{\text{III+Rb-NTP-}}$, pTA*rep*$^{\text{III-Rb+NTP+}}$ and pTA*r*ep$^{\text{III+Rb+NTP+ (AS)}}$ apparently abolished the replication of MSV-Kom. In these samples MSV-Kom replication was undetectable using 1 μg of total DNA for PCR amplification. The construct pTA*rep*$^{\text{III+Rb-NTP+}}$, carrying the mutation to the Rb-binding domain, enhanced the replication of MSV-Kom. The enhancement is clearly shown by comparing MSV-Kom controls (lanes 4 and 5) and the Rep wild type construct, pTA*rep*$^{\text{III+Rb+NTP+}}$ (lane 17), with pTA*rep*$^{\text{III+Rb-NTP+}}$ (lanes 13 and 14). The double mutant pTA*rep*$^{\text{III-Rb+NTP-}}$ (lanes 11 and 12) did not nhibit the replication of MSV-Kom although the mutant plasmid carrying the mutations to motif III abolished MSV-Kom replication. The mutations to the NTP/ATPase-helicase domain probably resulted in conformational changes that affected the oligomerization. This analysis demonstrated the transient *trans*-dominant effect of different Rep mutants on MSV-Kom replication in a rapidly dividing cell suspension system. To estimate the amount of MSV-Kom present in individual samples, replication specific quantitative (RSQ)-PCR was carried out (Figures 4.3).

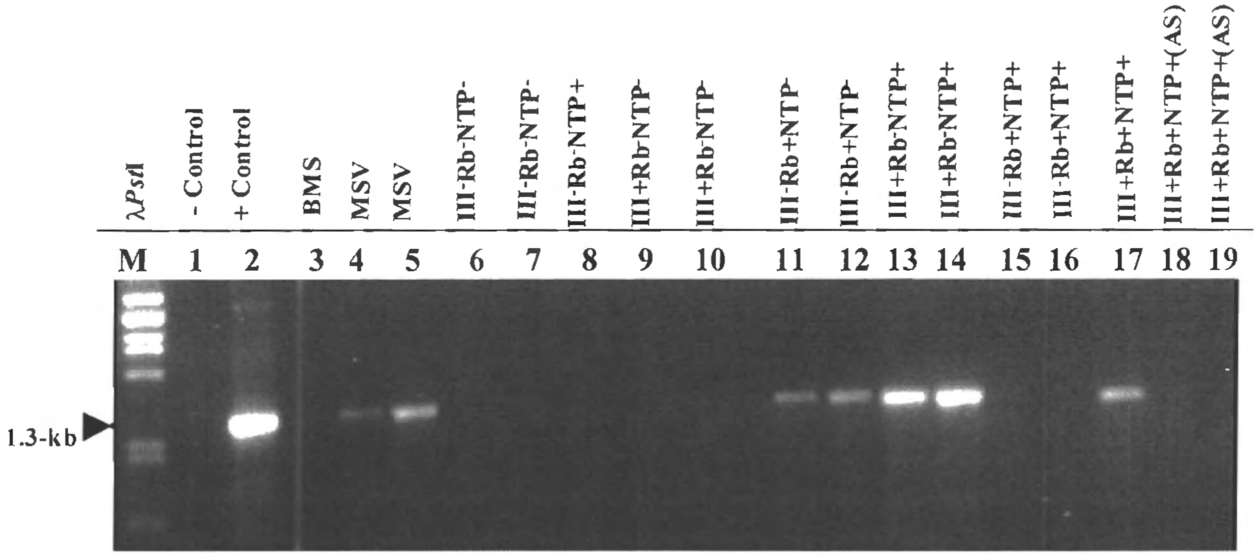

Figure 4.2. QPCR amplification of 1 μg of total DNA isolated from bombarded samples. The PCR products were separated on an ethidium bromide-stained 1.2% agarose gel. Lanes: M, λ-*Pst*I DNA markers; 1, control (water instead of DNA); 2, 10 ρg of pKEP177-*Pst*I; 3, BMS mock bombarded with water; 4-5, MSV-Kom (pKom602) only; 6-16, a mutant plasmid and MSV-Kom. Mutated and non-mutated domains are designated by $^-$ and $^+$ respectively. The actual amino acid changes to the targeted domains of the Rep protein are summarised in Table 3.1.

4.2.2 Quantitative PCR Analysis of the Inhibition of MSV-Kom Replication by *trans*-Dominant Rep Plant Vectors

Samples spiked with known amounts of pKEP177-*Pst*I (Figure 4.3 (A)) were amplified by PCR, potentially resulting in two products of the same size. They were differentiated by cleavage with *Pst*I, which cuts DNA amplified from pKEP177-*Pst*I and not from other MSV genomes (Figure 4.3 (B)). The individual band intensities were measured and the readings used to plot a standard curve (Figure 4.3 (C)). The amount of pKEP177-*Pst*I that was competitive with MSV-Kom replication products was estimated to be 1 x 10^{-2} ng. Individual competitive assays were carried out for plant vectors that supported MSV-Kom replication and the log ratios were used to extrapolate the amount of MSV-Kom DNA (results not shown). The results showed a six-fold increase in MSV-Kom replication in the presence of pTA*rep*$^{\mathrm{III+Rb-NTP+}}$ whereas MSV-Kom replication in the presence of pTA*rep*$^{\mathrm{III-Rb+NTP-}}$ was comparable with the wild type. The rest of the samples were not measured because replication of MSV-Kom was undetectable.

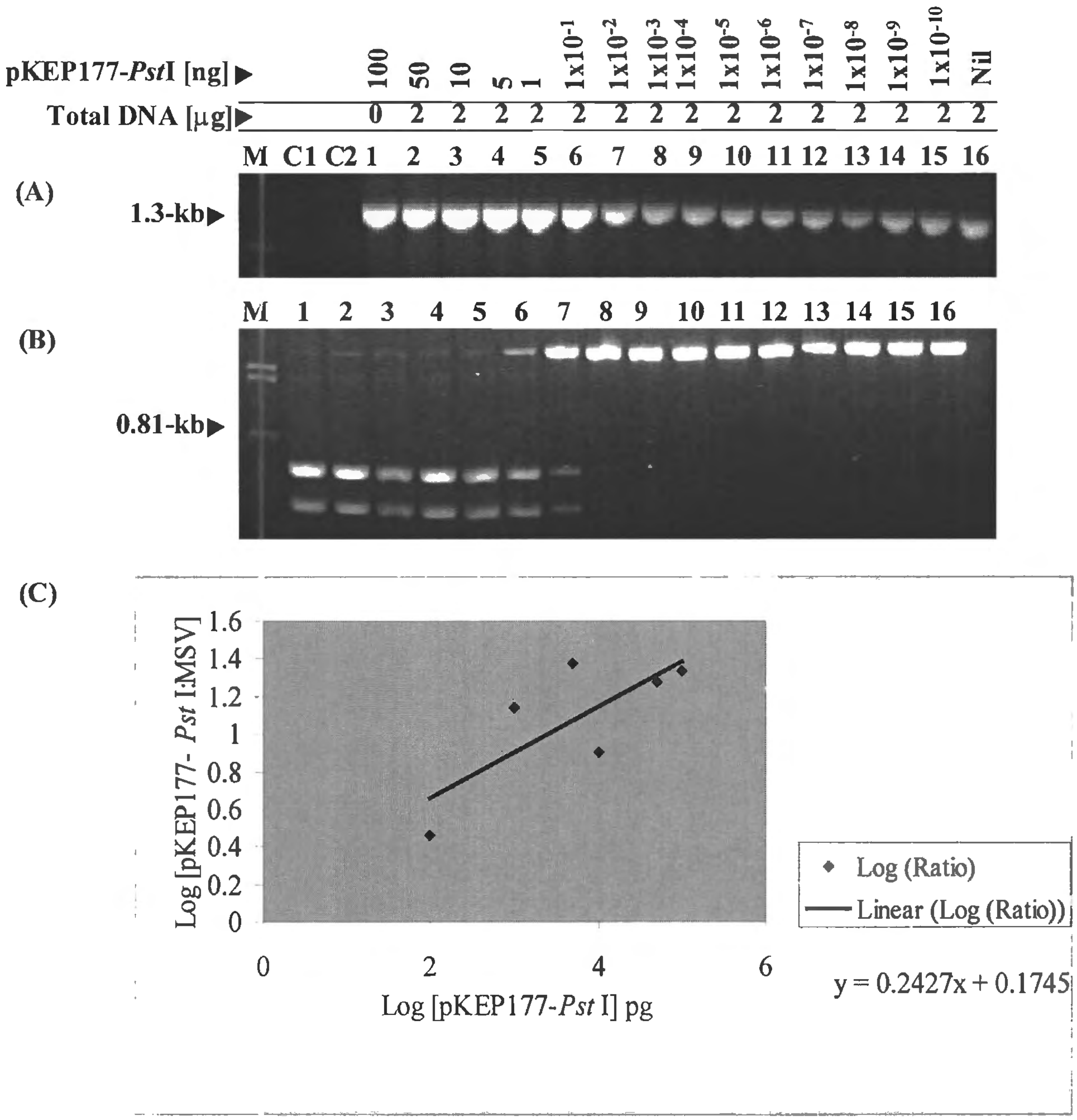

Figure 4.3. (A) Construction of a standard curve of RSQ-PCR co-amplified MSV-Kom Rep gene sequences using homologous mutant Rep of pKEP177-*Pst*I as an internal standard and wild type target Rep gene from replicating MSV-Kom as a competitor. For RSQ-PCR 2 µg aliquots of total DNA per sample were spiked with varying known amounts of pKEP177-*Pst*I prior to amplification. M, λ-*Pst*I molecular weight markers; C1 and C2, negative controls of water only. (B) Samples in lanes 1-16 are products of RSQ-PCR (panel (A)) cut with *Pst*I to differentiate the target from the internal standard

DNA. *Pst*I cuts the internal standard and not the wild type MSV-Kom Rep gene. Digested RSQ-PCR products were separated on an ethidium bromide-stained 1.2% agarose gel. Individual band intensities were measured using AlphaEase (stand alone program, Innotech Co-op USA) and the values were used to plot a standard curve. A positive control sample (MSV-Kom) of 2 µg of total DNA has an end-point of 1×10^{-2} ng of internal standard DNA. (C) Construction of a standard curve using RSQ-PCR products of MSV-Kom control samples spiked with known varying amounts of internal standard pKEP177-Ps*t*I. The logarithm of the ratio of pKEP177-*Pst*I and MSV-Kom band intensities derived from the experiment in (B) was plotted against the logarithm of the varying amounts of pKEP177-*Pst*I.

4.2.3 Southern Blot Assay for the Inhibition of MSV-Kom Replication in BMS Suspension Cells by *Trans*-Dominant Rep Mutants

Inhibition of MSV-Kom replication by mutant Rep plasmids was further analysed using Southern blots. There was correlation between QPCR and Southern analysis (Figures 4.2 and 4.4). Individual lanes were scanned using a densitometer and the results are shown in Figure 4.5. The replication of MSV-Kom resulted in two prevalent forms of replicative DNA namely the **c**ovalently **c**losed **c**ircular (**ccc**) DNA and **o**pen **c**ircular **s**ingle stranded (**ocs**) DNA. The Southern analysis results demonstrated that QPCR is a reliable and quick method for determining MSV-Kom replication in transient assay experiments.

DNA. *Pst*I cuts the internal standard and not the wild type MSV-Kom Rep gene. Digested RSQ-PCR products were separated on 1.2% ethidium bromide-stained agarose gel. Individual band intensities were measured using AlphaEase (stand alone program, Innotech Co-op USA) and the values were used to plot a standard curve. A positive control sample (MSV-Kom) of 2 μg of total DNA has an end-point of $1x10^{-2}$ ng of internal standard DNA. (C) Construction of a standard curve using RSQ-PCR products of MSV-Kom control samples spiked with known varying amounts of internal standard pKEP177-Ps*t*I. The logarithm of the ratio of pKEP177-*Pst*I and MSV-Kom band intensities derived from the experiment in (B) was plotted against the logarithm of the varying amounts of pKEP177-*Pst*I.

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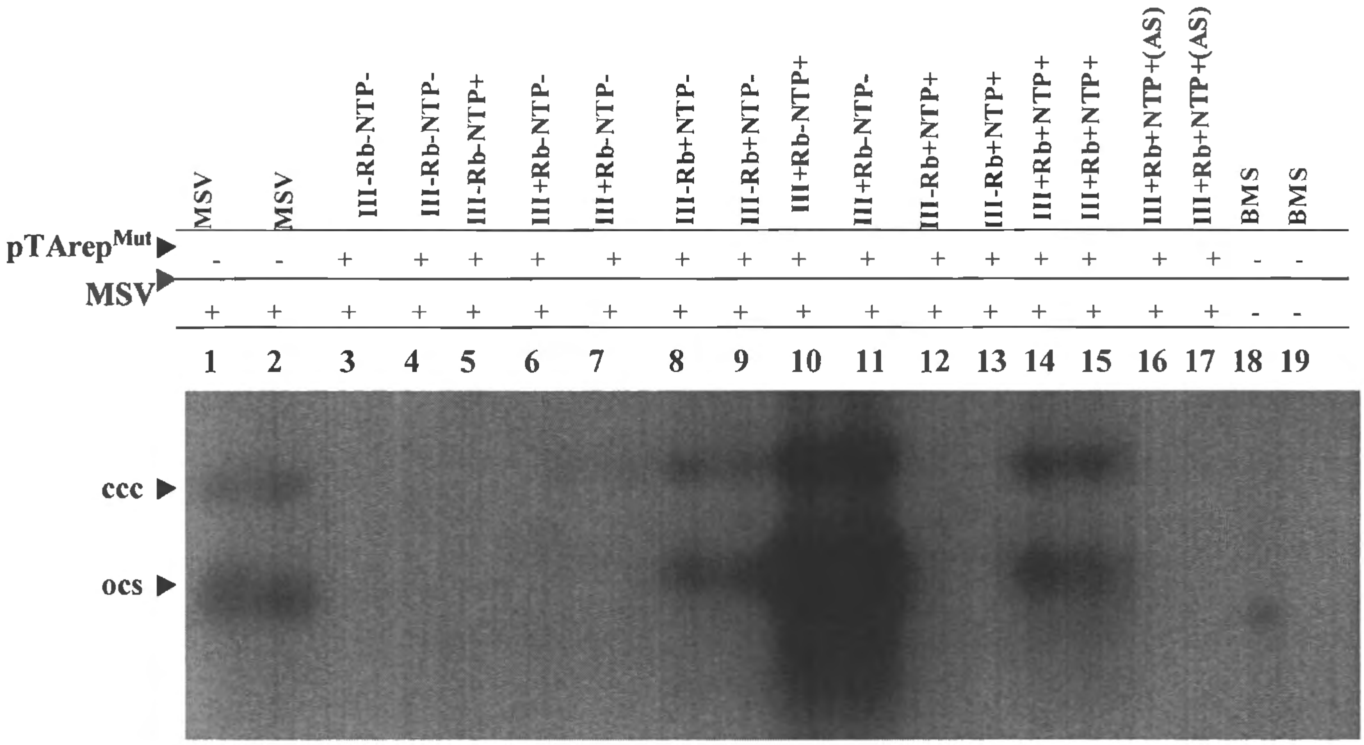

Figure 4.4. Ten μg of total DNA extracted 72 hr after bombardment was fractionated on a 1% ethidium bromide-stained agarose gel and used for Southern blot analysis. DNA was transferred to hybond N^{+} Amersham membrane as described in Materials and Methods. BMS samples were bombarded with MSV-Kom only (lanes 1, 2) and MSV-Kom + pTA*rep*$^{\text{Mut or AS}}$ (lanes 3-17) except for the control samples (lanes 18, 19). The entire Rep gene labelled with $[\alpha]$-^{32}P-dCTP was used as a probe. Mutated and non-mutated domains are designated by $^{-}$ and $^{+}$ respectively. The actual amino acid changes to the targeted domains of the Rep protein are summarised in Table 3.1.

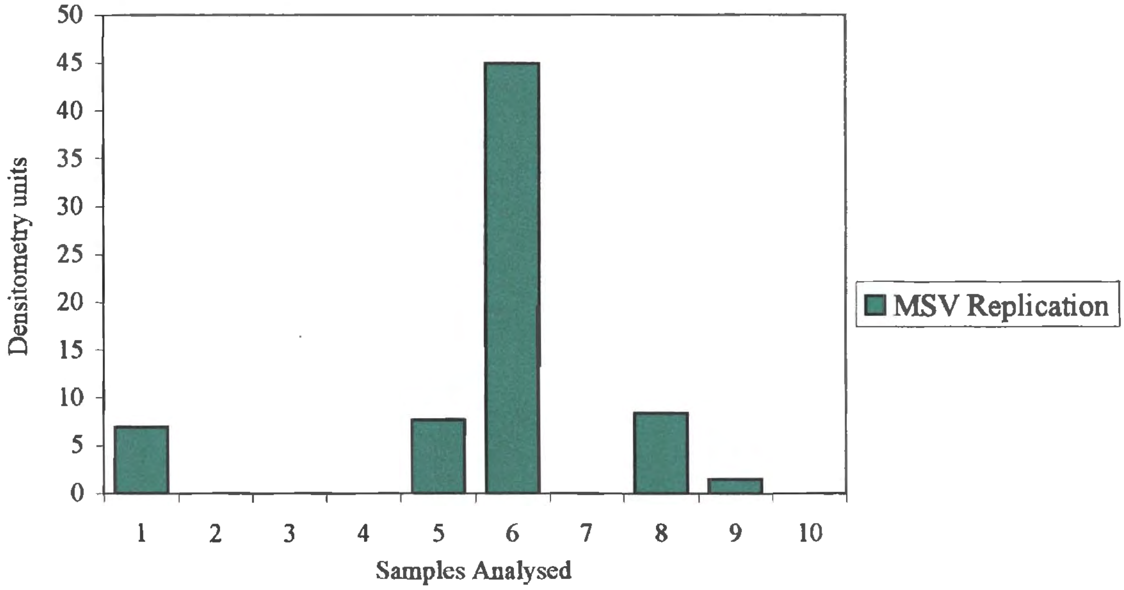

Figure 4.5. Densitometry readings of samples that showed replication when hybridized with a C1/C2 Rep gene probe. The total band intensity for each lane was measured. Samples: 1, MSV only; 2, pT*A*$rep^{\text{III-Rb-NTP-}}$; 3, pT*A*$rep^{\text{III-Rb-NTP+}}$; 4, pT*A*$rep^{\text{III+Rb-NTP-}}$; 5, pT*A*$rep^{\text{III-Rb+NTP-}}$; 6, pT*A*$rep^{\text{III+Rb-NTP+}}$; 7, pT*A*$rep^{\text{III-Rb+NTP+}}$; 8, pT*A*$rep^{\text{III+Rb+NTP+(S)}}$; 9, pT*A*$rep^{\text{III+Rb+NTP+(AS)}}$; 10, BMS mock bombardment.

In Figure 4.5, sample 1, containing MSV-Kom alone without Rep provided in *trans* was taken as the standard and the effects of the other samples were measured against it. In samples 2, 3, 4 and 7 replication of MSV-Kom was reduced to undetectable levels corresponding to 100% or complete inhibition. Sample 5 did not show any significant difference from sample 1, confirming the QPCR and RSQ-PCR data. A 6-fold increase in replication was evident in sample 6 that has a mutated Rb-binding domain, again confirming previous data. Sample 8, containing a wild-type Rep plasmid, did not have any effect on the replication of MSV-Kom.

4.3 DISCUSSION

The Rep protein of geminiviruses has several functions associated with RCR. These include directing the replication complex to the origin of replication (Fontes *et al.*, 1992; Thommes *et al.*, 1993), unwinding the dsDNA by helicase activity (Gorbalenya *et al.*, 1990; Hanson *et al.*, 1995) and the nicking-closing of DNA to initiate and terminate replication (Koonin and Ilyana, 1992; Stanley, 1995). In addition the Rep protein of TGMV, and the AC1 of BGMV and ACMV have been shown to autorepress their transcription by interacting with the upstream recognition sequences of the respective genes (Sunter *et al.*, 1993; Eagle *et al.*, 1994; Hong and Stanley, 1995; Orozoco *et al.*, 1997). Hong and Stanley (1996) reported the inhibition of ACMV in a transient expression system of *Nicotiana bethamiana* protoplasts when DNA A was co-transfected with wild-type AC1 and various mutant AC1 constructs. Inhibition of TYLCV using a 3'- truncated N-terminal portion of C1 (encoding the first 210 amino acids) in R_1 progeny of transgenic *Lycopersicon esculentum* cv Moneymaker was reported by Brunetti *et al.*, (1997) and in *Nicotiana bethamiana* (Noris *et al.*, 1996). However, the self-pollinated seeds from a highly resistant R_0 line expressing high levels of C1 failed to either germinate or grow properly to maturity. The R_1 seedlings grown either on agar plates or soil exhibited an altered phenotype linked to the "toxic" effects of an active transgene (T)-Rep protein. In this example the toxicity may be due to the interaction of an as yet unknown motif (s) with proteins associated with the development of the host.

Dominant negative mutants have been shown to effectively interfere with the replication of coliphage Q beta (Inokuchi and Hirashima, 1987) and adeno-associated virus (Chejanovsky and Carter, 1990) both of which use the RCR mechanism. To date no reports have been published on the use of dominant negative mutants of Rep or RepA as a strategy for developing resistance against mastreviruses.

Although transient expression experiments do not always accurately mimic the resistance mechanism operating in transgenic plants (Lomonossoff, 1995; Baulcombe, 1996) correlation has been demonstrated between tobacco protoplasts and transgenic plants (Hong and Stanley, 1996). They used a transient expression system to evaluate the efficacy of various mutant constructs to inhibit the replication of ACMV prior to producing transgenic plants. Our results show that the transient expression of various mutants of the Rep or RepA protein of MSV-Kom significantly reduced or abolished the replication of MSV-Kom in BMS cells. Mutants of MSV-Kom Rep or RepA were used to circumvent the possible "toxic" nature of Rep as was reported for TYLCV that resulted in some of the R_1 seedlings of *N. bethamiana* failing to germinate and others growing poorly (Brunetti *et al.*, 1997).

The use of a modified C1 of TYLCV to disrupt the function of wild type C1 was demonstrated in transgenic *N. bethamiana* plants (Norris *et al.*, 1996). The 3'-end of the C1 gene was truncated producing a portion of the N-terminus encoding 210 amino acids. The truncated protein retained among other functions DNA recognition and binding (Jupin *et al.*, 1995), cleavage and joining of the origin of replication (Heyraud-Nitschke *et al.*, 1995) but lacked the NTP-binding motif (Gorbalenya *et al.*, 1990). Resistance was evaluated by hybridization of young leaf tissues, leaf disc and protoplast assays. There was a correlation between the results obtained using the two different methods although the leaf disc and protoplast assays also demonstrated that resistance works at the single cell level. Evidence for a protein mediated rather than RNA-mediated resistance was also presented where TYLCV replication was inhibited using expression cassettes that were capable of transcribing and translating a truncated Rep gene. The cassette expressing a non-translatable truncated Rep mRNA transcript failed to confer resistance. The mode of resistance is based on competition between the transgene encoded Rep and the functional Rep synthesized by the incoming virus. Another likely mode of action is inhibition of infection through transcriptional repression of the functional viral C1 protein. The first mode of action is likely to be the case for inhibition of MSV-Kom replication by various mutants of full length Rep or RepA.

The basis of transdominance using a multifunctional regulatory protein is to alter the "secondary" functions of the protein without changing the "primary" functions. In this case the recognition, binding to the origin of replication of MSV-Kom and oligomerization of the Rep, RepA or RepA/Rep are the "primary" functions whereas the rest as described elsewhere in this thesis are "secondary". In mastreviruses, the translation of Rep mRNA results in the production of two proteins Rep and RepA, a process distinct from that of begomoviruses and curtoviruses. In TGMV, AL1 proteins form homodimers that may number up to eight. DNA cleavage and ligation are independent of AL1/AL1 interactions whereas AL1 DNA binding is dependent on the AL1/AL1 interaction (Orozco *et al.*, 1997). In MSV-Nigeria it was demonstrated that RepA interacts with Rep or with itself but Rep interacts weakly with itself (Liu *et al.*, 1999). The interaction leads to the formation of both homo- and hetero- dimers. This interaction is probably one of the modes of resistance manifested in the inhibition of MSV-Kom replication by various mutant constructs described in this thesis. The heterodimer is probably formed via the oligomerization domain and further interaction with other host factors for example Rb, leads to formation of dsyfunctional multimers

In plasmids that utilize the RCR, the concentration of Rep is maintained at a low, rate-limiting level and an increase in Rep has been shown to proportionally increase the replication frequency of the plasmids (Sogaard-Andersen *et al.*, 1984; Manch-Citron *et al.*, 1986; Swack *et al.*, 1987). Mastreviruses seem to use the same strategy although it is not clear whether Rep or RepA proteins are reused repeatedly or inactivated. The constitutive expression of mutants Rep$^{\text{III-Rb-NTP-}}$, $^{\text{III-Rb-NTP+}}$, $^{\text{III+Rb-NTP- and III-Rb+NTP+}}$ or the unspliced RepA equivalents probably resulted in unregulated binding of the mutants to the origin of replication of MSV-Kom and failure of the wild-type Rep to initiate replication. Alternatively mutant Rep or RepA proteins could oligomerize with wild type Rep or RepA, in a variety of configurations, making available fewer functional components of productive or active complexes. If the Rep or RepA function as a multimer, as has been shown for the functionally equivalent Reps of pT181 and TGMV (Thomas *et al.*, 1990; Rasooly *et al.*, 1994; Orozco *et al.*, 1997), the constitutive expression of various mutants of Rep or RepA could affect MSV-Kom replication by disturbing the equilibrium between monomeric, heteromeric and multimeric forms. Interactions of Rep↔RepA* (where *denotes mutant), RepA*↔Rep, Rep*↔Rep and RepA↔Rep* probably result in non-productive interactions with the viral origin of replication.

The mutant Rep$^{\text{III-Rb+NTP-}}$ failed to abolish the replication of MSV-Kom, although the mutant Rep$^{\text{III-Rb+NTP+}}$ completely inhibited it. The Chou-Fasman (1978) structure of Rep$^{\text{III-Rb+NTP-}}$ around the Rb-binding domain is the same as that of the wild type. The lack of inhibition is therefore probably due to decreased unproductive interaction between the wild type Rep or RepA and mutant Rep or RepA leading to more active wild-type complexes.

An unexpected result was the six-fold enhancement of MSV-Kom replication by the mutant $\text{Rep}^{\text{III+Rb-NTP+}}$ carrying mutations N^{201}→L and $\mathbf{E}^{202}$→K in the **LLCNE** motif. Chou-Fasman predicted secondary structures showed that mutants $\text{Rep}^{\text{III-Rb+NTP+}}$ and $\text{Rep}^{\text{III+Rb-NTP+}}$ are the same although $\text{Rep}^{\text{III-Rb+NTP+}}$ completely abolished replication of MSV-Kom. Viral oncoproteins associated with replication mimic the interaction between cyclins and Rb that enables the phosphorylation of Rb by Cdks and concomitant inactivation of the Rb. It is difficult to account for the Rb-mutant Rep supporting MSV-Kom replication in cells where ZmRb1 is absent or is at very low levels. The BMS cells unlike differentiated cells are permissive of viral replication and it is conceivable to speculate that MSV-Kom replication does not need Rb. The apparent enhancement of replication of MSV-Kom can not be satisfactorily explained by the interaction between mutant and wild type Rep leading to unproductive complexes. The results showed that MSV-Kom replication in rapidly dividing cells does not require an intact **LLCNE** Rb-binding domain of Rep or RepA. The behaviour of the Rb mutant is the subject of further research although it is likely to be involved in transmission of the virus by the insect *C. mbila* (Rybicki, personal communication).

Monomers of Rep or RepA with wild type motif III are capable of nicking DNA at the conserved nonanucleotide *in vitro* without binding to the origin of replication. The double mutant of RepA or $\text{Rep}^{\text{III+Rb-NTP-}}$ probably inhibits the replication of MSV-Kom by competing with the wild type for the origin of replication. The mutant presumably nicks dsDNA and fails to unwind the duplex. The effect of mutating the NTP alone was not tested but can be inferred by comparing the effects of Rep $^{III+Rb-NTP-}$ and Rep$^{III+Rb-NTP+}$.

The results have clearly demonstrated the *trans*-inhibitory effect of some constructs under stringent virus challenge. An interesting aspect is resistance conferred by simple one-amino acid substitution or amino acids flanking a conserved residue for example the conserved tyrosine in the nicking-closing motif of RCR replicons. The work reported here is one of the first to describe the use of full-length mutant Rep or RepA as a resistance strategy against a mastrevirus, MSV-Kom. My expectations are that the mutants I introduced in some of the Rep or RepA functional domains are probably going to solve the "altered-phenotype" problem. It is reasonable to suspect that the toxicity of geminiviruses transgene-Rep proteins is possibly due to its interaction with the Rb. The Rep↔Rb interaction has also been demonstrated in TGMV whose Rep does not have the **LxCxE** Rb-binding motif. As has been demonstrated mostly in begomoviruses, it is possible to obtain resistance against MSV-Kom using minimally but strategically modified Rep or RepA proteins. Work on the molecular diagnosis of the effect(s) of the Rb-mutant on virus transmission by the leafhopper, *C. mbila* is underway. The mutants that completely abolished MSV-Kom replication have been selected for transformation into MSV-Kom susceptible *Digitaria sanguinalis* for *in planta* resistance studies.

CHAPTER FIVE

CONTENTS

PAGE

CHAPTER FIVE

The Inhibition of Maize Streak Virus Replication in Black Mexican Sweetcorn Suspension Cells by the Transient Expression of Antisense mRNA

ABSTRACT

Mastreviruses replicate to high copy numbers in the nucleus of an infected host cell using the Rep protein to initiate and terminate the RCR. The multifunctional Rep and RepA proteins are encoded by the spliced C1:C2 or unspliced C1, respectively, the mRNA of which is expressed as a rare transcript making it a suitable candidate for antisense inhibition. Antisense mRNA directed at the analogous AC1 or C1 has been reported to be effective against TGMV or TYLCV, members of the genus *Begomovirus*. I report the inhibition of MSV-Kom replication, a member of the genus *Mastrevirus*, using antisense mRNA to the entire C1:C2 ORF in BMS suspension cells. The results demonstrate that the level of inhibition of MSV-Kom replication correlates with the strength of the promoter used.

5.0 INTRODUCTION

Antisense RNA is known to be an effective means of regulating gene expression in both prokaryotes (Inouye, 1988; Simons, 1988) and eukaryotes (Krol *et al.*, 1988). There are many models to account for antisense RNA inhibitory mechanisms, most of which assume that RNA forms a duplex with its complementary mRNA: this could interfere with transcription, the sense RNA:antisense RNA duplex could be rapidly degraded by double-stranded RNA-specific nucleases (Blomberg *et al.*, 1990; Krinke and Wulff, 1990), and/or the duplex may not be transported from the nucleus to the cytoplasm. Degradation and/or failure of the duplex to reach the cytoplasm would inhibit the translation of target mRNA (Smith *et al.*, 1988; Cornelissen, 1989; Cornelissen and Vandewiele, 1989). Antisense RNA is also effective against multigene copies and also acts in *trans,* making it ideal for the suppression of viral infection.

In plant pathology, work on the use of antisense has focused on RNA viruses that replicate in the cytoplasm and in most of these cases antisense RNA has not been particularly successful (Baulcombe *et al.*, 1987; Cuozzo *et al.*, 1988; Hemenway *et al.*, 1988; Rezaian *et al.*, 1988; Powell *et al.*, 1989; for review, see Hackland *et al.*, 1993). The likely reason for these failures is the choice of the coat protein (CP) gene as the target and limited possibilities for antisense-mRNA interaction in the cytoplasm (Cuozzo *et al.*, 1988; Hemenway *et al.*, 1988; Powell *et al.*, 1989). The CP gene is expressed at a higher level than other genes in most viruses and is not absolutely required for viral replication. There are exceptions, however, and Kawchuk *et al.*, (1991) successfully obtained potato plants resistant to *Potato leafroll virus* (PLRV) by expressing both the sense and antisense RNA to the CP; Hackland *et al.*, 2000) obtained tobacco plants resistant to tobacco necrosis virus via CP gene antisense expression.

Geminiviruses are serious plant pathogens that infect important food crops but little has been done to engineer resistance to these viruses. To date antisense RNA to the Rep gene has been successfully used in tobacco plants against TGMV (Day *et al.*, 1991) and TYLCV (Bendahmane and Gronenborn, 1997). The antisense of TGMV also protected against BCTV infection (Bejarano and Lichtenstein, 1994). The development of resistance to MSV infection by the use of antisense RNA is an attractive prospect because of the small genome variation between different African isolates (2.0-2.2%; Briddon *et al.*, 1994; Rybicki, 1994). The quasispecies nature of MSV (Isnard *et al.*, 1998) potentially presents a problem of resistance-breaking isolates: antisense RNA is probably the best method to solve this because it is effective against target sequences with as low as 55% overall identity, or dissimilar genomes with small patches of high sequence identity (Bejarano and Lichtenstein, 1994).

The use of transient expression of antisense RNA as a means of testing whether the system works was demonstrated using *Cherry leafroll virus* (CLV) CP in tobacco protoplasts (Brooks and Bruening, 1995), but has not yet been used for assaying mastrevirus replication inhibition. Experiments presented here, using the BMS transient expression system I have developed, demonstrate the use of PCR analysis to assess the ability of three plasmids bearing antisense Rep sequences to interfere with MSV-Kom replication.

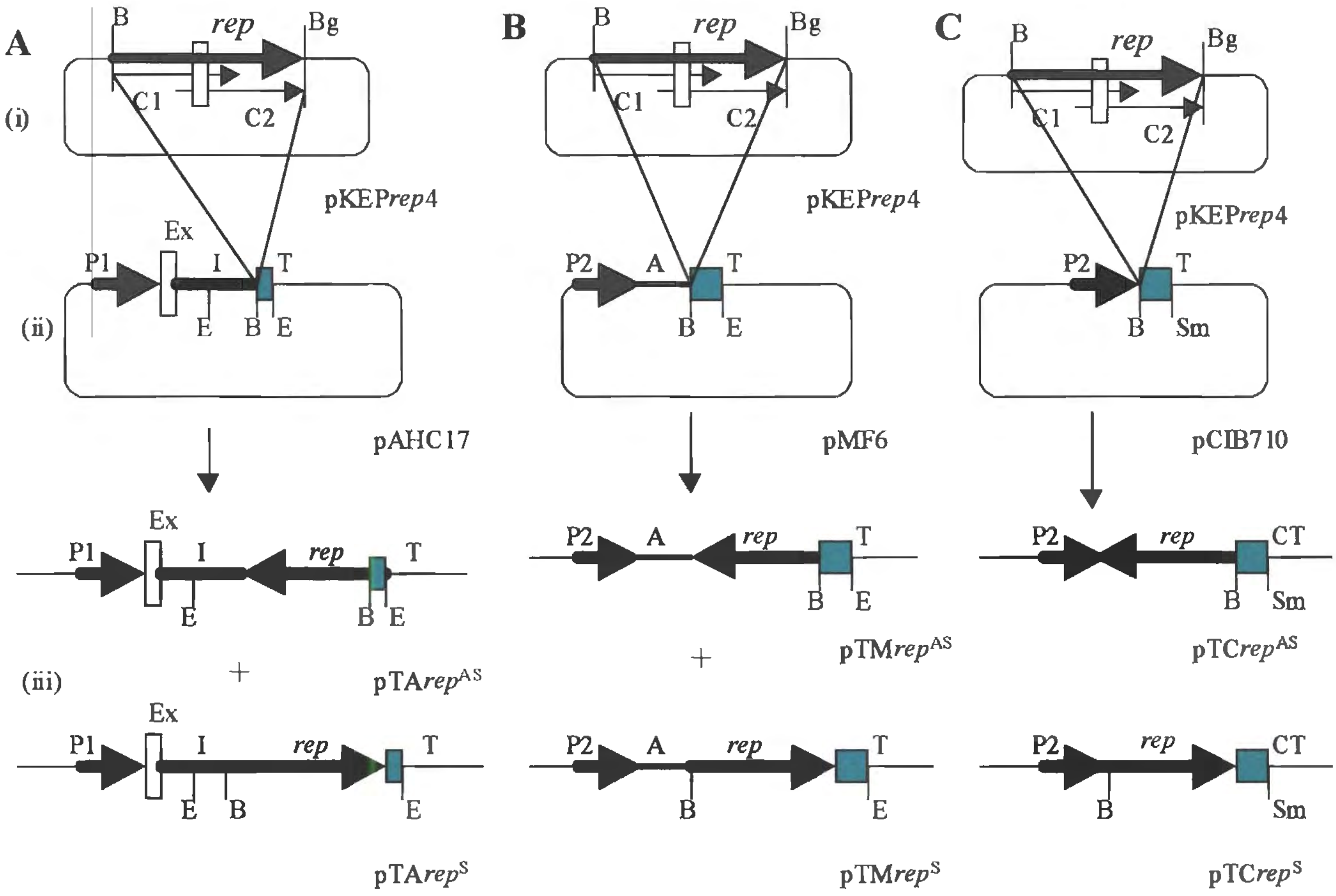

Figure 5.1. MSV Rep protein sequences used in the BMS suspension cell transient expression system. The Rep gene was cloned into plant vectors, carrying different promoters, in both orientations, antisense (AS) and sense (S). The Rep gene encodes the C1 and C2 ORFs. Abbreviations: P1, ubiquitin promoter; Ex, exon of the ubiquitin gene; I, intron; T, NOS 3' (nopaline synthase gene) terminator; P2, CaMV35S promoter; A, alcohol dehydrogenase gene intron 1; CT, CaMV35S RNA terminator; B, *Bam*HI; E, *Eco*RI; Sm, *Sma*I; Bg, *Bgl*II.

5.1 MATERIALS AND METHODS

5.1.1 DNA Manipulations

Restriction enzymes and DNA modifying enzymes were purchased from Boehringher Mannheim, Amersham and New England Biolabs. Chemicals for bacteria and plant tissue cultures were from Difco and Sigma. Standard DNA manipulation techniques were used as described in Sambrook *et al.*, (1989).

5.1.2 Plasmid Constructions

The plasmids are shown in Figure 5.1. pTA*rep*^{AS} and pTA*rep*^{S} contain the MSV Rep gene in the antisense and sense orientation respectively, driven by the ubiquitin promoter. They were obtained by cloning a *Bam*HI-*Bgl*II fragment from pKEP*rep*4 (Palmer, 1997) into the *Bam*HI site of plasmid pAHC17 (Cornejo *et al.*, 1993). Appropriate restriction endonucleases were used to map the sense pTA*rep*^{S} and antisense pTA*rep*^{AS} orientation of the Rep gene relative to the promoter. Plasmid pTM*rep*^{AS} contains the Rep gene driven by the *Cauliflower mosaic virus* (CaMV)-35S promoter coupled to an alcohol dehydrogenase 1 (*Adh*1) intron (Callis *et al.*, 1987). It was obtained by cloning the same *Bam*HI-*Bgl*II fragment into the *Bam*HI site of plasmid pMF6 (Callis *et al.*, 1987). Both sense pTM*rep*^{S} and antisense pTM*rep*^{AS} plasmids were mapped using appropriate restriction enzymes. Plasmid pTC*rep*^{AS} contains the Rep gene driven by the CaMV35S promoter. It was obtained by cloning the *Bam*HI-*Bgl*II fragment into the *Bam*HI site of plasmid pCIB710 (Rhostein *et al.*, 1987). Appropriate restriction enzymes were used to map the sense pTC*rep*^{S} and antisense pTC*rep*^{AS} orientation of the Rep gene.

Plasmids pKom602 (a 1.1-mer of MSV-Kom) and pKEP177-*Pst*I were provided by Drs W Schnippenkoetter (Schnippenkoetter, 1998) and K Palmer (Palmer, 1997) respectively. All the plasmids were amplified in *Escherichia coli* strain DH5α and purified by Nucleobond AX® plasmid isolation kits.

5.1.3 Microprojectile Bombardment

Bombardment conditions were as described by Gordon-Kamm *et al.*, (1991). Fifty µl of gold particles (1 µm) suspended in 50% glycerol (60 mg ml^{-1}) were combined with varying concentrations of plasmid DNA and precipitated. The precipitation mix contained 50 µl of 2.5 M $CaCl_2$ and 20 µl of 0.1 M spermidine. The mix was incubated on ice for 10 min and vortexed for 5 min. The pellet was washed twice with absolute ethanol, resuspended in 60 µl of 100% ethanol and divided for six shots. Plasmid concentrations ranging from 1-5 µg were used for each single precipitation. BMS cells were bombarded using the Helium Gene Delivery System (Bio-Rad) at 650 psi. Each bombardment experiment comprised two duplicate shots and bombardments were repeated three times.

5.1.4 Transient Expression Assays

For each transient assay, BMS cells from four to five day cultures were filtered onto sterile Whatman no.1 filter paper and incubated on MS medium (Murashige and Scoog, 1962) containing 12% sucrose for 12 hr prior to bombardment. The BMS cell samples were incubated in the dark at 25°C and total DNA was isolated 72 hr after bombardment.

5.1.5 Inhibition of MSV Replication by Antisense Rep genes

Each plasmid shown in Figure 5.1 (iii) was bombarded together with pKom602 (MSV-Kom), which releases infectious monomeric genomes of MSV, into BMS cells which were incubated in the dark for 72 hr at 25°C. Replication was measured by QPCR (Zachar *et al.*, 1993) using the replicative form-specific primers described in Chapter 3.

5.1.6 Qualitative PCR Analysis

DNA isolated from the bombarded BMS cells was amplified using MSV-Kom replicative form-specific degenerate primers (MSVQPCR1 and MSVQPCR2) described in Chapter 3. The PCR procedure outlined in Figure 4.1 generates a fragment of 1.3- kb.

5.1.7 Replicative form-Specific quantitative (RSQ)-PCR analysis

To estimate the amount of replicative form MSV-Kom DNA in the bombarded samples, I used RSQ-PCR as described by Zachar *et al.*, (1993) and illustrated in Figure 4.1, panel 3. This involved spiking the positive control DNA samples (competitor) with serial dilutions of pKEP177-*Pst*I to generate a standard curve. Forty ng of total DNA from the positive control (pKom602) samples was spiked with varying amounts of pKEP177-*Pst*I. The mix was amplified using PCR primers MSVQPCR1 and MSVQPCR2. The serial dilutions of the internal standard, pKEP177-*Pst*I, ranged from 10 pg to 1×10^{-6} pg (Figure 5.3, panel A(i)). The PCR fragments were differentiated by restriction of the co-amplified PCR products with *Pst*I that cuts the fragment generated from the template pKEP177-*Pst*I and not MSV (Figure 5.3, panel A(ii)).

The digested PCR products were separated on an ethidium bromide-stained 1.2% agarose gel. Individual band intensity readings were measured using UviBand software for Windows 95 (UVItech, St John's Innovation Centre Cowley Road, Cambridge CB4 4WS, England) and the values were used to plot a standard curve (Figure 5.3 panel B). Forty ng of individual samples were spiked with a known concentration of the internal standard and amplified using the same PCR procedure. The log of the ratio of band intensities of internal standard:competitor was calculated (Figure 5.3 panel C (i), (ii) and (iii)). The amount of MSV target sequences in the total DNA sample was extrapolated from the standard curve (Figure 5.3 pannel B).

5.2 RESULTS

5.2.1 Analysis of the Inhibition of MSV Replication by Plant Vectors Utilising Different Promoters

The three pairs of plasmids, AS and S, shown in Figure 5.1 were evaluated for their ability to interfere with MSV replication using a BMS suspension cell transient expression system. The *rep* genes in the sense and antisense constructs pTA*rep*^{S} and pTA*rep*^{AS}, pTM*rep*^{S} and pTM*rep*^{AS}, pTC*rep*^{S} and pTC*rep*^{AS}, were placed under the control of the ubiquitin plus first exon and first intron, cauliflower mosaic virus 35S plus *alcohol dehydrogenase* 1 intron, and cauliflower mosaic virus 35S promoters, respectively. The antisense constructions were designed to transcribe mRNA sequences complementary to that of the MSV Rep mRNA.

To subject the antivirus strategy to strong MSV-Kom challenge, equal amounts of antisense and MSV-Kom plasmids were biolistically introduced into BMS suspension cells at the same time, as opposed to introducing the virus 12 hr later

The digested PCR products were separated on a 1.2% ethidium bromide stained agarose gel. Individual band intensity readings were measured using UviBand software for Windows 95 (UVItech, St John's Innovation Centre Cowley Road, Cambridge CB4 4WS, England) and the values were used to plot a standard curve (Figure 5.3 panel B). Forty ng of individual samples were spiked with a known concentration of the internal standard and amplified using the same PCR procedure. The log of the ratio of band intensities of internal standard:competitor was calculated (Figure 5.3 panel C (i), (ii) and (iii)). The amount of MSV target sequences in the total DNA sample was extrapolated from the standard curve (Figure 5.3 pannel B).

5.2 RESULTS

5.2.1 Analysis of the Inhibition of MSV Replication by Antisense-Expressing Vectors Utilising Different Plant Promoters

The three pairs of plasmids, AS and S, shown in Figure 5.1 were evaluated for their ability to interfere with MSV replication using the BMS suspension cell transient expression system. The Rep rep gene s in the sense and antisense constructs pTA*rep*^{S} and pTA*rep*^{AS}, pTM*rep*^{S} and pTM*rep*^{AS}, pTC*rep*^{S} and pTC*rep*^{AS}, were placed under the control of the ubiquitin plus first exon and first intron, cauliflower mosaic virus 35S plus *alcohol dehydrogenase* 1 intron, and cauliflower mosaic virus 35S promoters, respectively. The antisense constructions were designed to transcribe mRNA sequences complementary to that of the MSV Rep mRNA.

To subject the antivirus strategy to strong MSV-Kom challenge, equal amounts of antisense-expressing and MSV-Kom-releasing plasmids were biolistically introduced into BMS suspension cells at the same time, as opposed to introducing the virus 12 hr later

(Brooks and Bruening, 1995). Total DNA was isolated 72 hr after bombardment and MSV-Kom replication was analysed using qualitative PCR. The introduction of the antisense constructs, pTA*rep*^{AS} and pTM*rep*^{AS}, significantly reduced MSV-Kom replication (Figure 5.2; lanes 8 and 9, 10 and 11). The construct pTC*rep*^{AS} with the Rep gene under the control of CaMV35S alone did not show any detectable reduction when compared with the control (Figure 5.2; lanes 12 and 13 compared with lanes 6 and 7).

The sense constructs were tested to check if the gene of interest was being expressed by complementation experiments using replication-incompetent MSV-Kom (results not shown). Although the gene of interest was expressed, the expression levels were not measured. Taking into account the high stringency conditions used to evaluate the efficacy of the antiviral strategies, the reduction in MSV-Kom replication was significant.

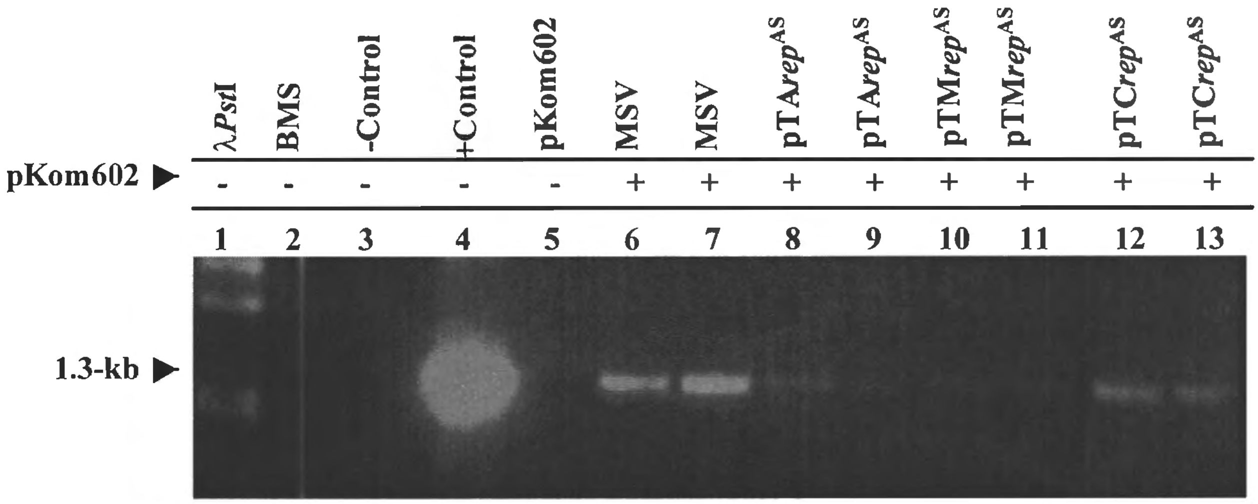

Figure 5.2. QPCR analysis of the replication of MSV-Kom in the presence of an antisense transcript of C1:C2 mRNA transcribed using promoters of different strengths. Forty ng of total DNA were used for PCR amplification. Lanes: 1, λ*Pst*I DNA marker; 2, mock bombarded BMS cells; 3, water in place of DNA; 4, 10 ng of plasmid pKEP177-*Pst*I; 5, 10 ng of plasmid pKom602 (not in total DNA from the BMS cells therefore not replicatively released); 6 and 7, samples bombarded with plasmid pKom602 alone; 8 and

9, pTA*rep*^{AS} plus pKom602; 10 and 11, pKom602 plus pTM*rep*^{AS}; 12 and 13, pTC*rep*^{AS} plus pKom602.

5.2.2 Estimation of the level of Inhibition of MSV-Kom Replication by Plant Vectors Utilising Different Promoters

To enable the RSQ-PCR analysis of multiple samples a calibration curve was constructed to standardize the interpolation of the concentration of other samples (Figure 5.3 panel C). The concentration of the internal standard pKEP177-*Pst*I was subjected to end-point dilution (Figure 5.3 panel A) as opposed to subjecting both templates to end-point dilution (Wang and Mark, 1990). To estimate the amount of MSV in 40 ng of total DNA from samples co-bombarded with MSV-Kom and antisense constructions (Figure 5.2 lanes 8-13), individual samples were spiked with a known amount of pKEP177-*Pst*I and subjected to RSQ-PCR (Figure 5.3 panel C).

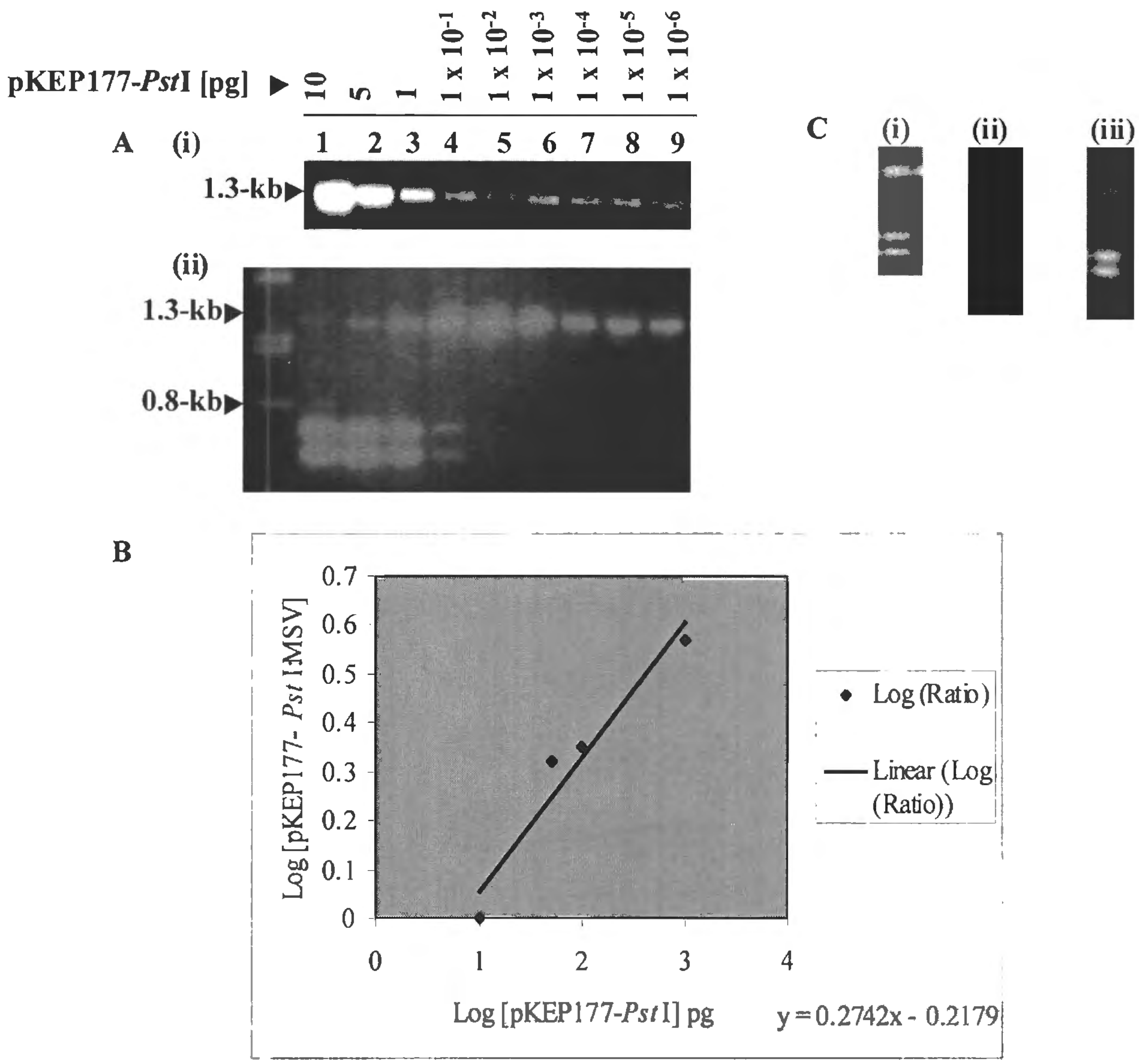

Figure 5.3. A (i) Amplification of standard amounts of wild-type MSV-Kom template DNA and internal standard pKEP177-*Pst*I using QPCR. The serial dilutions of pKEP177-*Pst*I ranging from 10 pg to $1x10^{-6}$ pg were co-amplified with 40 ng of total DNA from samples transfected with MSV-Kom. The PCR products in A (i) were digested with *Pst*I to differentiate those from MSV-Wt and pKEP177-*Pst*I and fractionated on 1.2% ethidium bromide-stained agarose gel, panel A (ii). B) Construction of a calibration curve from the data obtained by RSQ-PCR and digital image processing of the electrophoretic pattern in A (ii). C) Amplification of 40 ng of DNA samples from (i) pTA*rep*^{AS} plus MSV, (ii) pTM*rep*^{AS} plus MSV-Kom and (iii) pTC*rep*^{AS} plus MSV-Kom each spiked with 5 pg of pKEP177-*Pst*I. Individual band intensities were measured using UviBand software 1998 and the log ratio of pKEP177-*Pst*I:MSV-Kom was used to interpolate the individual sample concentration.

The amount of MSV-Kom in each of the transfected samples was estimated by digital image processing of the electrophoretic patterns in Figure 5.3 C (i), (ii) and (iii) and interpolation from the calibration curve (Figure 5.3 panel B). The amounts were compared to the control sample to establish any significant differences. The level of MSV-Kom in pTC*rep*^{AS} and control samples was five times more than that in pTA*rep*^{AS} and pTM*rep*^{AS}. The amounts estimated by QPCR for pTA*rep*^{AS} and pTM*rep*^{AS} did not show any significant differences.

The results showed a marked reduction in MSV-Kom replication proportional to the strength of the promoter used to express the *rep* gene. There was no difference between samples transfected with constructs pTA*rep*^{AS} and pTM*rep*^{AS} in which the *rep* gene is under the ubiquitin and CaMV35S-*Adh*1 intron promoters. The replication of MSV-Kom in samples transfected with pTC*rep*^{AS} under the CaMV35S promoter was not significantly different from the control samples transfected with MSV-Kom alone.

5.3 DISCUSSION

This research describes a transient BMS expression system using different antisense RNA cassettes designed to inhibit the replication of MSV-Kom. The antisense RNA cassettes used promoters that varied in strength of expression. The rationale for this approach was that if MSV-Kom replicates to very high copy numbers, as does the closely related mastrevirus WDV (Timmermans *et al.*, 1992), greater repression of the targeted Rep gene activity will be required to inhibit its replication. If this is not done the residual Rep activity will support viral replication. The target of the antisense RNA is the rare messenger RNA of the Rep or RepA protein encoded respectively by the spliced

C1:C2 or C1 gene (Wright *et al.*, 1997). The Rep or RepA protein of MSV-Kom is a multifunctional protein just like the Rep or RepA of MSV-Nigeria (Horvath *et al.*, 1998). In addition the expression of Rep or RepA is regulated by splicing of the 92-bp intron (Wright *et al.*, 1997). All the essential functions of Rep or RepA and the splicing of the C1:C2 mRNA to produce Rep make it an ideal target for obtaining virus resistance. The expression of the Rep RNA as an "antisense-messenger" will subvert the deleterious effects that the constitutive synthesis of active Rep protein probably has on the plant cell (Norris *et al.*, 1996; Brunnetti *et al.*, 1997; Bendahmne and Gronenborn, 1997).

The Rep antisense plasmids pTA*rep*^{AS} and pTM*rep*^{AS} significantly reduced the replication of MSV-Kom compared to plasmid pTC*rep*^{AS}. The only difference between these plasmids is the strength of the individual promoters used to express the "antisense-messenger". Although the ubiquitin promoter used in plasmid pTA*rep*^{AS} is one of the best known for the expression of genes in monocots, the level of inhibition was comparable to that of plasmid pTM*rep*^{AS} that used the CaMV35S-*Adh*1 intron promoter to express the "antisense-messenger". The coupling of an the *Adh* 1 intron to the CaMV35S is known to increase gene expression levels by 10-fold in cultured maize cells (Callis *et al.*, 1987). The probable reason for the two plasmids having the same effect is that for the inhibition of MSV-Kom to occur, the level of the "antisense-messenger" must reach a certain threshold level. The CaMV35S is not a strong promoter for the expression of genes in monocots compared to the ubiquitin and CaMV35S-*Adh*1 promoters. Even in dicots where the CaMV35S promoter is supposedly strong, it is normally fused with upstream single or double enhancers to increase the expression levels (Day *et al.*, 1991; Bejarano and Lichtenstein, 1994; Bendahmane and Gronenborn, 1997). The plasmid pTC*rep*^{AS} did not have an inhibitory effect on MSV-Kom replication because the "antisense-messenger" RNA levels were probably below the threshold level necessary to prevent the residual Rep protein from initiating replication.

Although we did not measure the levels of antisense RNA a strong positive correlation was observed between the strength of the promoter used and the level of inhibition. The advantage of using the transient expression system is that there is no variation in gene expression due to positional effects. The only variation is that of biolistic transfection from one experiment to another. In this experiment the variation was not significant between transfections. The mechanism for the inhibition could not be inferred from the results but it may involve the formation of an RNA-RNA duplex. This duplex may be degraded by nucleases or be trapped in the nucleus or the target mRNA is not available for translation.

The transient expression system we applied was very stringent in that it did not allow for the expression of the "antisense-messenger" in advance of MSV-Kom infection. MSV-Kom and the "antisense-messenger" were co-introduced into BMS cells in a situation that did not mimic that in transgenic plants where the expression of the "antisense-messenger" occurs prior to infection. The poor inhibition afforded by pTC*rep*^{AS} is possibly not a true reflection of how the plasmid will perform when stably integrated in a host plant. My results of the level of MSV-Kom inhibition estimated by RSQ-PCR may therefore underestimate the likely effectiveness of this strategy in transgenic plants because of the high stringency used. Golemboski *et al*., (1990) and Carr *et al*., (1992) demonstrated that their protoplast transient expression system underestimated the potential of the replicase gene to inhibit replication of TMV and CMV in transgenic plants.

Antisense RNA technology has been used to demonstrate resistance to geminiviruses of the genus *Begomovirus* and *Curtovirus* (Day *et al.*, 1991; Bejarano and Lichtenstein, 1990; Bendahmane and Gronenborn, 1997). My results suggest that this strategy may find application in the control of diseases caused by viruses of the genus *Mastrevirus*.

CONCLUSION AND FUTURE WORK

Many examples of pathogen-derived resistance against geminiviruses have been demonstrated in experimental plants (mostly *Nicotiana tabacum* or *N. bethamiana*) and not in the natural host plants for the viruses. However, this does not imply that the results obtained are not important for the development of an effective resistance strategy. There are a few examples where PDR has been demonstrated in the natural host, such as the CP-transgenic tomato plants resistant to TYLCV-Is (Kunik *et al.*, 1994). In addition, the *Bean dwarf mosaic virus* (BDMV) BV1 and BC1-transgenic tomato plants expressing the mutated or wild type protein manifested a "symptom-delay" phenotype when inoculated with a related virus ToMoV (Hou *et al.*, 2000). Although the resistance involved the use of heterologous transgenes (derived from BDMV instead of ToMoV), ToMoV is well adapted to the host plant (tomato) used.

In this work I used two systems that are as close to maize as is possible, namely BMS suspension cells and Jubilee sweetcorn maize plants. The advantage of using these systems as described in Chapters 3, 4 and 5 is that reasonable information on the introduced mutants is obtained prior to maize transformation. An inherent problem with the use of Reps is that abnormal and or infertile plants could be produced. In the case of resistance to ToMoV, the mutants to the conserved amino acids in the BDMV BV1 and BC1 ($K^{95}E$ and $D^{78}N$) produced phenotypically normal plants that were infertile. The two mutants manifested abnormal flower development (BV1-$K^{95}E$) and failure to recover R1 plants (BC1-$D^{78}N$) (Hou *et al.*, 2000). It is tempting to assume that the use of truncated proteins will solve this problem. However this might not be the case as the truncated Rep of TYLCV and the inadvertent truncated BC1 of BDMV produced typical infection phenotypes in their respective experimental hosts. In the search for a solution to these problems associated with the Rep strategy, the use of various combinations of mutations that do not affect primary functions (origin specificity, binding, and oligomerization) is reasonably attractive.

My results demonstrate that the Rb-binding motif has a probable role to play in symptom development as the mutated construct produced attenuated symptoms in maize plants. It is therefore conceivable to use mutant combinations that include the Rb-binding motif mutant for resistance against MSV-Kom. The hypothesis is that such mutants will have less impact on the developmental processes of the transgenic plant due to the de-linking of the mutant Rep with the plant cell cycle. Except for the single Rb-mutant, all the mutant combinations abolished the replication of MSV-Kom. The antisense mRNA also inhibited replication in a promoter-dependent manner.

In my view, it is critical to investigate why the Rb- mutant enhanced the replication of MSV-Kom in BMS cells, where the environment is already supportive of viral replication. In BMS cells, the levels of PCNA are high, indicative of active DNA synthesis and replication. More insight into this mutant could be obtained by carrying out binding assays, including pull-down assays, in BMS suspension cells. This might indicate which, if any, host factors interact with the Rb- mutant. Another interesting line to pursue is that of investigating the effect of successive leafhopper transmissions of the mutant with a view to determine whether or not it reverts to the wild type or mutates to any other form. Another area for further investigation is the *myb*-like domain within the NTP\ATPase-helicase domain. In plants the Myb protein is involved in the regulation of many developmental genes, some of which control seed formation and development. This domain might be the clue to all the phenotypic problems associated with the expression of Rep in plants.

In conclusion, work is currently in progress to produce transgenic maize plants expressing the various mutants described in this thesis. My expectations are that transgenic plants with varied characteristics will be produced, opening the way for a detailed study of the impact of the MSV-Kom Rep on plant development. If taken from the point of view of plant development, in addition to virus resistance, the results of this thesis hold even greater promise.

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