CHARACTERISATION OF DNA REPLICATION OF TOMATO LEAF CURL GEMINIVIRUS

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To my wife, Maryam

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"Characterisation of DNA replication of tomato leaf curl geminivirus"

ABSTRACT

Tomato leaf curl geminivirus (TLCV) causes a severe disease of tomato in the northern parts of Australia. In addition to the wild-type strain of TLCV, three distinct virus-like agents were identified in samples from Darwin, Northern Territory. Two of these were found to be new strains of TLCV, referred to as the Darwin 1 (D1) and Darwin 2 (D2) strains. They had an overall nucleotide sequence similarity with the TLCV type strain of approximately 95%, but their sequence similarity with the type strain within the origin of replication (*ori*) was only 80%.

The third disease agent, unlike the TLCV strains, was mechanically transmissible and caused initial stunting of infected tomato plants followed by shoot elongation and hairlessness. This disease agent was isolated and shown to be a circular RNA. The complete nucleotide sequence of this 356 nucleotide RNA revealed it to be a sequence variant of potato spindle tuber viroid.

The biological relatedness of the TLCV strains was studied by *in vivo* complementation experiments. Mixed agroinfection experiments in whole plants indicated that the D1 and type strains can replicate simultaneously in the same host, however, the D1 strain could not complement a replication-defective mutant of the type strain. This indicated that each strain replicated independently in co-infected plants.

TLCV type and D1 strains were also examined *in vitro* for cross interaction with the replication-associated protein (Rep), the only viral protein required for viral DNA replication. The TLCV type strain Rep was expressed in *E. coli* and purified to

homogeneity. Electrophoretic mobility shift assays (EMSA) demonstrated that the purified Rep binds specifically to a 120 bp fragment within the TLCV intergenic region. Fine analysis of the binding regions within the 120 bp fragment, using DNase I footprinting, demonstrated two footprints covering the sequences GCAATTGGTGTCTCTCAA and TGAATCGGTGTCTGGGG containing a direct repeat of the motif GGTGTCT (underlined). The results suggest that the repeated motif is involved in virus-specific Rep binding, but may not constitute the entire binding element. This is the first demonstration of geminivirus sequence elements involved in Rep binding by direct protein-DNA interaction assays. Using EMSA and DNase I footprinting it was further observed that the Rep of the type strain does not bind to the *ori* of the D1 strain demonstrating that the Rep-binding elements within the TLCV *ori* are strain-specific.

In addition to the full-length viral DNAs, subgenomic DNA molecules of varying sizes were also found in plants infected with TLCV. Two discrete subgenomic DNAs derived from each of the TLCV type and D1 strains were cloned and fully sequenced and found to contain large deletions which disrupt most or all of the viral genes. However, in all cases the viral *ori* including the Rep binding domains was intact. The subgenomic DNAs were also shown to be encapsidated by TLCV coat protein. Co-agroinfection of leaf strips with constructs of viral and subgenomic DNA repeats indicated that subgenomic DNAs replicated only in the presence of the TLCV DNA and that interfered with the replication of the viral genome. The D1 strain could not support the replication of subgenomic DNAs derived from the TLCV type strain indicating that the replication of each subgenomic DNA is completely dependent on the presence of its cognate Rep protein.

PUBLICATIONS

Behjatnia, S. A. A., Dry, I. B., Krake, L. R., Condé, B. D., Connelly, M. I., Randles, J. W., and Rezaian, M. A. (1996). New potato spindle tuber viroid and tomato leaf curl geminivirus strains from a wild *Solanum* sp. *Phytopathology* 86: 880-886.

Behjatnia, S. A. A., Dry, I. B., and Rezaian, M. A. (1998). Identification of the replication associated protein binding domain within the intergenic region of tomato leaf curl geminivirus. *Nucleic Acids Res.* (In press).

STATEMENT

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university. To the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference has been made in the text.

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Seyyed Ali Akbar Behjatnia

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ABBREVIATIONS

The abbreviation of geminiviruses are outlined in Table 1.1.

ATP	Adenosine-triphosphate
ATPase	Adenosine-triphosphatase
bp	Base pair
BSA	Bovine serum albumin
dATP	2′-Deoxy-adenosine-5′-triphosphate
dCTP	2′-Deoxy-cytosine-5′-triphosphate
dGTP	2′-Deoxy-guanosine-5′-triphosphate
dNTP	Mixture of deoxyribonucleoside-triphosphate in equimolar concentrations
dTTP	2′-Deoxy-thimidine-5′-triphosphate
DDT	Dithiothreitol
DNase	Deoxyribonuclease
ds	Double-stranded
EDTA	Ethylenediamine *tetra* acetic acid
EMSA	Electrophoretic mobility shift assay
g	Gram
g	Gravity
GTPase	Guanosine-triphosphatase
IR	Intergenic region
IPTG	*iso*-propyl-β-D-thiogalactopyranoside
kbp	Kilo base pair
kDa	Kilo Dalton

l	Litre
Mr	Molecular weight
M	Molar
mA	Milli Ampere
n	Nano
nt	Nucleotide
ORF	Open reading frame
ori	Origin of replication
PCR	Polymerase chain reaction
Rep	Replication-associated protein
RF	Replicative form
rpm	Revolutions per minute
RNase	Ribonuclease
SDS	Sodium dodecyl sulphate
SG	Subgenomic
ss	Single-stranded
TEMED	N, N, N′- N′-Tetramethylethylenediamine
Tris	Tris(hydroxymethyl)methylamine (Tris base)
U	Unit
UV	Ultra-violet
v/v	Volume per volume
w/v	Weight per volume
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

Chapter One

General Introduction

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1.1 Introduction

Geminiviruses are a unique group of plant viruses with significant agricultural importance particularly in tropical and subtropical regions. The name geminivirus was first coined by Harrison *et al.* (1977) to describe viruses comprising small quasi isometric particles (Latin, *gemini* = twins) and containing circular ssDNA. Geminiviruses have been placed in the family *Geminiviridae* by the International Committee on the Taxonomy of Viruses (ICTV) in 1995 (Briddon and Markham, 1995).

Geminiviruses contain either one (monopartite) or two (bipartite) circular DNA molecules, are transmitted by either leafhoppers or whiteflies and infect a wide variety of monocotyledonous or dicotyledonous plants. In addition to their significance as pathogens, these viruses provide an excellent model system for the study of DNA replication and gene expression in plants. They also provide a challenge for developing control measures by genetic modification of target crops.

Considerable progress has been made toward understanding the molecular biology and pathology of geminiviruses, and several excellent reviews report this progress (Davies and Stanley, 1989; Laufs *et al.*, 1995a; Lazarowitz, 1992; Stanley, 1985; Timmermans *et al.*, 1994). In this chapter, an overview of the classification, genome organisation and function of geminiviruses is provided, with emphasis on the geminiviruses causing leaf curl disease in tomato.

1.2 Taxonomy of *Geminiviridae*

The geminiviruses are subdivided into three subgroups or genera according to their biological and molecular characteristics including host range, type of insect vector, and the number of genomic DNA molecules (Briddon and Markham, 1995; Rochester *et al.*, 1993; Stanley, 1985). Members of subgroup I infect monocotyledonous hosts and are transmitted by leafhoppers in a persistent manner. The type member of this subgroup is maize streak virus (MSV). Subgroup II has only one well-characterised virus, beet curly top virus (BCTV), which infects dicotyledonous hosts. Like subgroup I, BCTV is transmitted persistently by a leafhopper. Members of subgroups I and II have a single genomic DNA component. Subgroup III is the largest subgroup and is represented by bean golden mosaic virus (BGMV) as the type member. Subgroup III members have either a monopartite or bipartite genome, infect dicotyledonous hosts, and are transmitted by the whitefly *Bemisia tabaci* (Gennadius) in a persistent manner.

The last ICTV list of the *Geminiviridae* (Briddon and Markham, 1995) includes 11 species and two tentative species in subgroup I, one species and two tentative species in subgroup II, and 49 species and 9 tentative species in subgroup III (Table 1.1).

Table 1.1 *List of species and tentative species in the Geminiviridae.*

Virus	Abbreviation	Genomic sequence accession number
Genus "subgroup I geminivirus"		
Species		
Bromus striate mosaic virus	BrSMV	
chloris striate mosaic virus	CSMV	M020021
Digitaria streak virus	DSV	M23022
Digitaria streak mosaic virus	DiSMV	
Maize streak virus*	MSV	X01089, X01633, X94330
Miscanthus streak virus	MiSV	D00800, D01030
Panicum streak virus	PanSV	X60168
Paspalum striate mosaic virus	PSMV	
sugarcane streak virus	SSV	M82918
tobacco yellow dwarf virus	TYDV	M81103
wheat dwarf virus	WDV	X02869
Tentative species		
bajra streak virus	BaSV	
chickpea chlorotic dwarf virus	CpCDV	
Genus "subgroup II geminivirus"		
Species		
beet curly top virus*	BCTV	U02311, X04144
Tentative species		
tomato leafroll virus	TLRV	
tomato pseudo-curly top virus	TPCTV	
Genus "subgroup III geminivirus"		
Species		
Abutilon mosaic virus	AbMV	X15983, X15984
Acalypha yellow mosaic virus	AYMV	
African cassava mosaic virus	ACMV	X17095, X17096, J02058, J02057
Ageratum yellow vein virus	AYVV	
Asystasia golden mosaic virus	AGMV	
bean calico mosaic virus	BCaMV	L27264, L27266
bean dwarf mosaic virus	BDMV	M88179, M88180
bean golden mosaic virus*	BGMV	M91604, M91605, L01635, L01636, M10070, M10080, D00200, D00201, M88686,
Bhendi yellow vein mosaic virus	BYVMV	
Chino del tomato virus	CdTV	
cotton leaf crumple virus	CLCrV	
cotton leaf curl virus	CLCuV	
Croton yellow vein mosaic virus	CYVMV	
Dolichos yellow mosaic virus	DoYMV	
Eclipta yellow vein virus	EYVV	
Euphorbia mosaic virus	EuMV	
honeysuckle yellow vein mosaic virus	HYVMV	
horsegram yellow mosaic virus	HgYMV	
Indian cassava mosaic virus	ICMV	Z24758, Z24859
Jatropha mosaic virus	JMV	

Table 1.1 (continued)

Virus	Abbreviation	Genomic sequence accession number
limabean golden mosaic virus	LGMV	
Malvaceous chlorois virus	MCV	
melon leaf curl virus	MLCV	
Macrotyloma mosaic virus	MaMV	
mungbean yellow mosaic virus	MYMV	D14703, D14704
okra leaf curl virus	OLCV	
pepper huasteco virus	PHV	X70418, X70419
pepper mild tigre virus	PepMTV	
potato yellow mosaic virus	PYMV	D00940, D00941
Pseuderanthemum yellow vein virus	PYVV	
Rhynchosia mosaic virus	RhMV	
Serrano golden mosaic virus	SGMV	
sida golden mosaic virus	SiGMV	
squash leaf curl virus	SqLCV	M38182, M38183,M63155 to M63158
Texas pepper virus	TPV	
tobacco leaf curl virus	TLCV	
tomato golden mosaic virus	TGMV	K02029, K02030
tomato leaf curl virus - Au	ToLCV-Au[1]	S53251
tomato leaf curl virus - In	ToLCV-In[1]	L12739, L11746
tomato mottle virus	TMoV	L14460, L14461
tomato yellow dwarf virus	ToYDV	
tomato yellow leaf curl virus - Is	TYLCV-Is	X15656
tomato yellow leaf curl virus - Sr	TYLCV-Sr	X61153, Z25751, L27708
tomato yellow leaf curl virus - Th	TYLCV-Th	M59838, M59839
tomato yellow leaf curl virus - Ye	TYLCV-Yem	X79429
tomato leaf crumple virus	TLCrV	L27267 to L27269
tomato yellow mosaic virus	ToYMV	
watermelon chlorotic stunt virus	WmCSV	X79403
watermelon curly mottle virus	WmCMV	
Tentative species		
cowpea golden mosaic virus	CpGMV	
eggplant yellow mosaic virus	EYMV	
Eupatorium yellow vein virus	EpYVV	
lupin leaf curl virus	LLCV	
papaya leaf curl virus	PaLCV	
sida yellow vein virus	SiYVV	
Solanum apical leaf curl virus	SALCV	
soybean crinkle leaf curl virus	SCLV	
Wissadula mosaic virus	WiMV	

*Type species

[1]The abbreviation TLCV has been used for tomato leaf curl virus throughout this work.

Phylogenetic studies based on nucleotide sequence comparisons support the classification of geminiviruses into three genera. The relationship among 36 different geminiviruses (Padidam *et al.,* 1995a) is shown as a phylogenetic tree in Fig. 1.1. This comparison has shown that subgroup I and subgroup III geminiviruses form two distinct clusters, irrespective of the part of the genome considered. Of the two leafhopper-transmitted viruses that infect dicots, tobacco yellow dwarf virus (TYDV) has a sequence most similar to subgroup I viruses. The genome organisation of beet curly top virus (BCTV) is similar to subgroup III viruses, but its coat protein sequence is more similar to subgroup I viruses (Stanley *et al*., 1986).

The phylogenetic analysis of geminiviruses (Fig. 1.1) displays distinct branches within subgroup I and subgroup III viruses. The subgroup III viruses form clusters according to their geographical origin, with distinct branches for New World and Old World regions (Padidam *et al.,* 1995a).

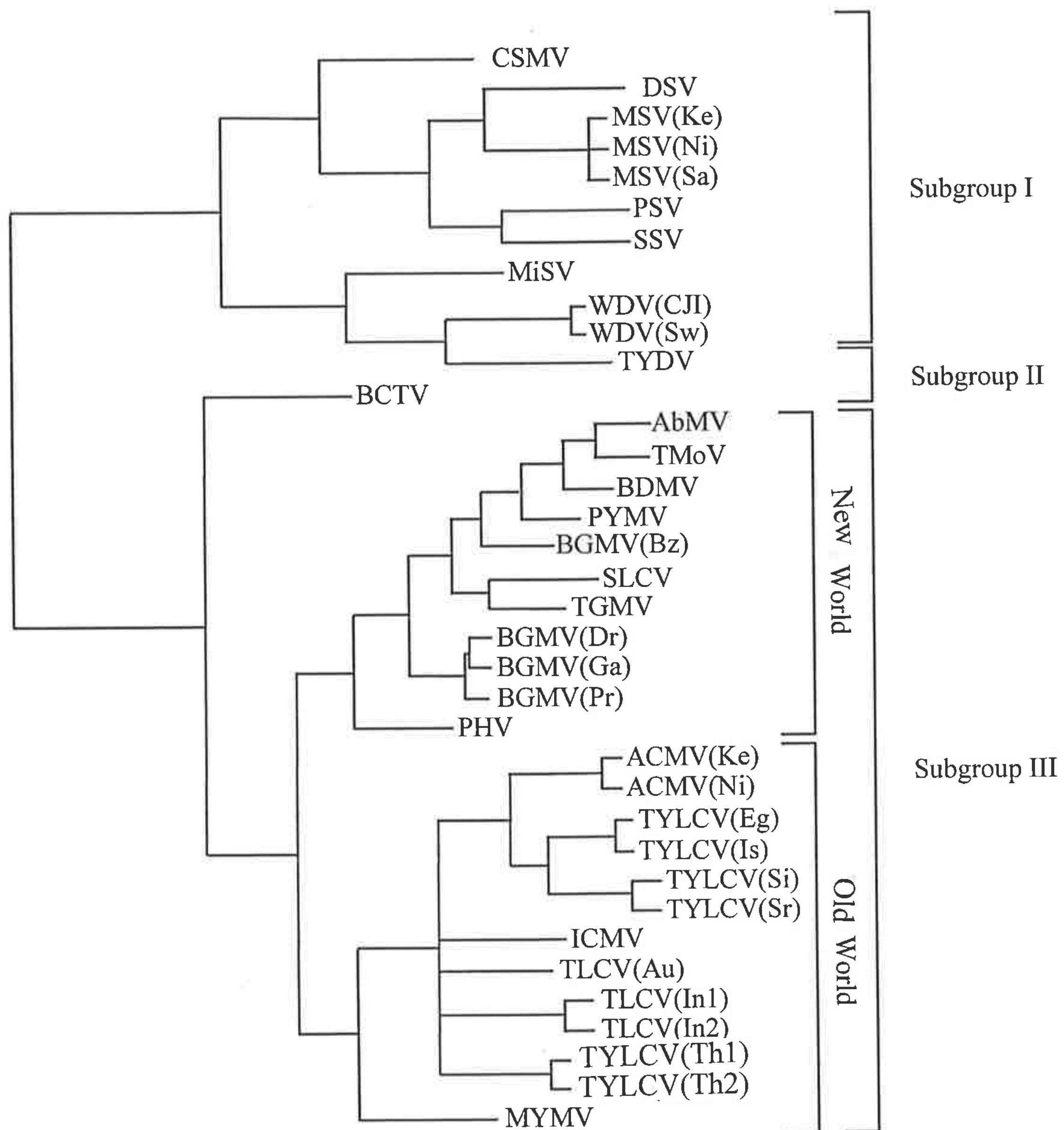


Fig. 1.1 Phylogenetic tree obtained from the alignment of nucleotide sequences of 36 geminiviruses showing the relationship between these viruses (reproduced from Padidam et al., 1995a).

1.3 Particle morphology and composition

The main characteristic of the *Geminiviridae* is twinned isometric particles of approximately 18 × 30 nm (Briddon and Markham, 1995; Matthews, 1991). They consist of two incomplete icosahedra (T=1) and sediment at approximately 70 *S*. Virions contain a single coat protein species with *Mr* of 28-34 x 10^3. A virion consists of 22 morphological units (capsomeres) each estimated to contain 5 coat protein molecules. The viral particle contains a single molecule of circular single-stranded DNA of 2.5-3.0 kbp in size (Briddon and Markham, 1995; Matthews, 1981). The virus particles are efficient immunogens (Matthews, 1981). In plants, virus particles accumulate in the nucleus, producing large aggregates (Goodman, 1981; Osaki and Inouye, 1981).

1.4 Genome organisation of geminiviruses

The genome of geminiviruses contains only a small number of genes arranged on both DNA strands. The genes carry the information for replication, systemic movement, induction of disease symptoms, encapsidation and transmission by insects (Hormuzdi and Bisaro, 1993; Lazarowitz, 1992; Navot *et al.*, 1991; Stanley *et al.*, 1986; Stenger, 1994). The genes are separated by non-coding intergenic regions. The intergenic region (or common region in bipartite geminiviruses) is different in sequence between different geminiviruses. An inverted repeat capable of forming a hairpin structure and containing the nonanucleotide motif 5′-TAATATTAC-3′ within the loop of the hairpin structure is common to all geminiviruses.

1.4.1 Subgroup I viruses

The single component genome of leafhopper-transmitted monopartite geminiviruses (subgroup I) contains four ORFs, two each in the virion-sense strand (V1

and V2) and complementary-sense strand (C1 and C2) (Fig. 1.2A). In addition to a large noncoding intergenic region (LIR) located between the 5′ ends of the V1 and C1 ORFs, a small noncoding intergenic region (SIR) also exists between the 3′ ends of the V2 and C2 ORFs (Fig. 1.2A). The presence of a small complementary-sense primer-like molecule, bound to the genome within the small intergenic region, has been shown for some species of subgroup I geminivirus including CSMV (Andersen *et al.*, 1988), DSV (Donson *et al.*, 1987), MSV (Donson *et al.*, 1984; Howell, 1984), TYDV (Morris *et al.*, 1992) and WDV (Hayes *et al.*, 1988).

1.4.2 Subgroup II viruses

The single component genome of BCTV contains seven ORFs, three in the virion-sense and four in the complementary-sense strand (Fig. 1.2B). The organisation of the BCTV genome and its gene products closely resemble the DNA A of the whitefly-transmitted subgroup III viruses (see below and Fig 1.2). An exception is the coat protein, which is more closely related to those of the leafhopper-transmitted subgroup I viruses (Stanley *et al.*, 1986). Another unique feature is the presence of three functional virion-sense genes in the BCTV genome (Hormuzdi and Bisaro, 1993).

1.4.3 Subgroup III viruses

The majority of subgroup III geminiviruses have a bipartite genome consisting of two circular DNA components (A and B). The DNA A and DNA B of any given bipartite geminivirus show no sequence similarity except for a common intergenic region of about 200 nucleotides (Fig. 1.2C). The DNA A component contains four ORFs, one in the virion- and three in the complementary-sense strand (Fig. 1.2C). They are required for viral DNA replication and encapsidation of progeny virions. The DNA

B component contains two ORFs, one in each strand (Fig. 1.2C), which are required for viral systemic movement within the infected plant. Both genomic components of bipartite geminiviruses are required for infectivity (Lazarowitz, 1992; Stanley, 1983). However, it has been observed that agroinoculation of plants with only DNA A of the Thai isolate of tomato yellow leaf curl geminivirus produces systemic movement and symptoms in inoculated plants (Rochester *et al.,* 1990).

A number of whitefly-transmitted subgroup III viruses contain only a single DNA genome. These monopartite genomes contain six ORFs (Fig. 1.2D). The two virion-sense ORFs are arranged in a fashion similar to those of the subgroup I geminiviruses, whereas the arrangement of the four complementary-sense ORFs is similar to that of the DNA A components of bipartite subgroup III geminiviruses (Antignus and Cohen, 1994; Dry *et al*., 1993; Kheyr-Pour *et al*., 1991; Navot *et al*., 1991; Noris *et al*., 1994). In contrast to subgroup I viruses, no complementary-sense primer-like molecule has been found in the subgroup III geminiviruses (Davies and Stanley, 1989; Stanley and Townsend, 1985).

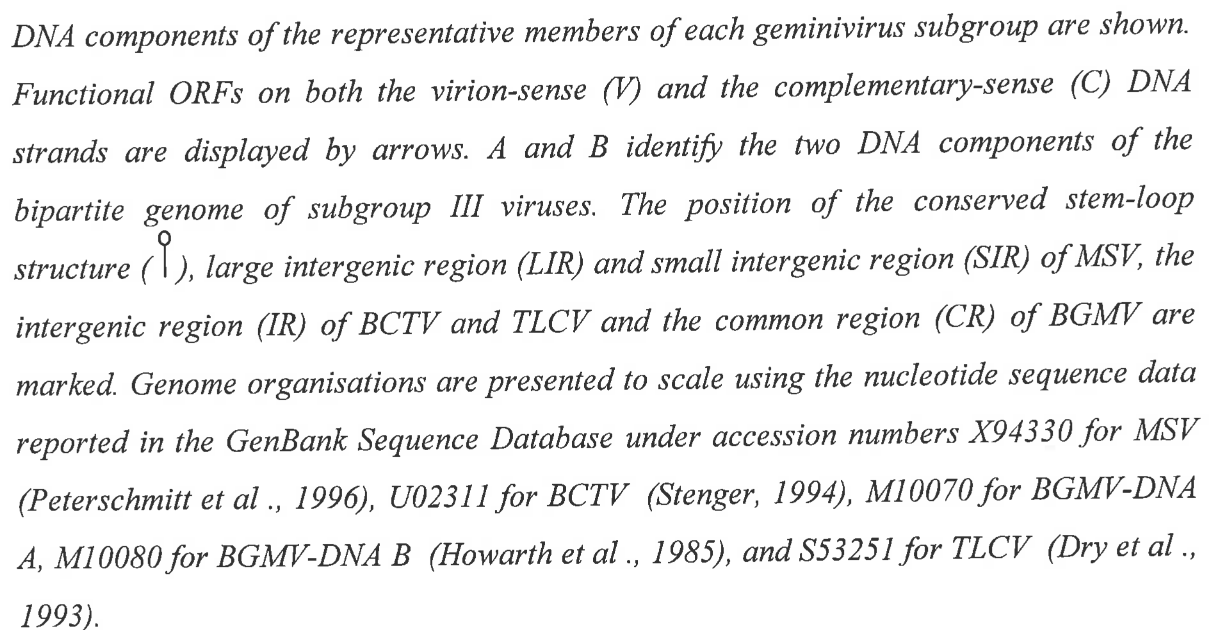
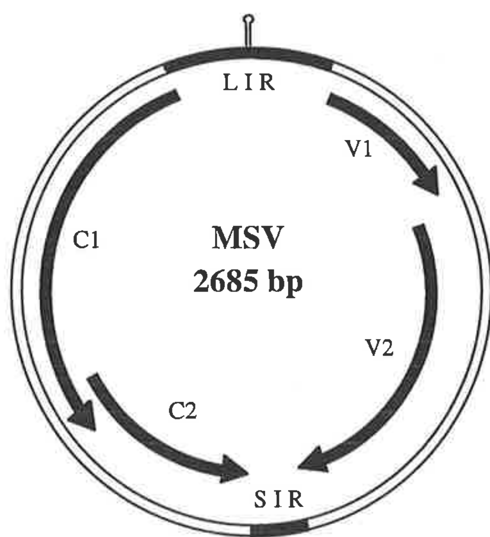
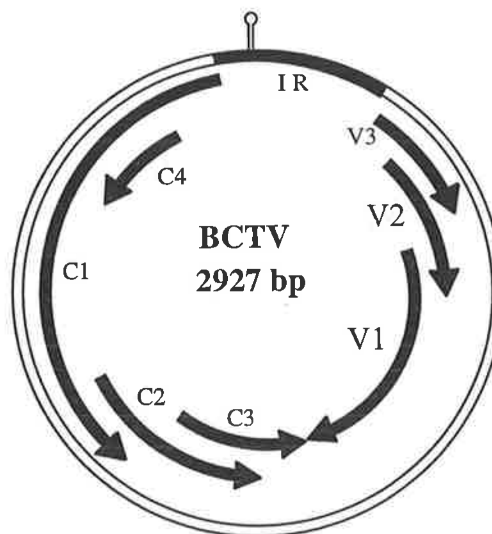


Fig. 1.2 *Genome organisation of geminiviruses*

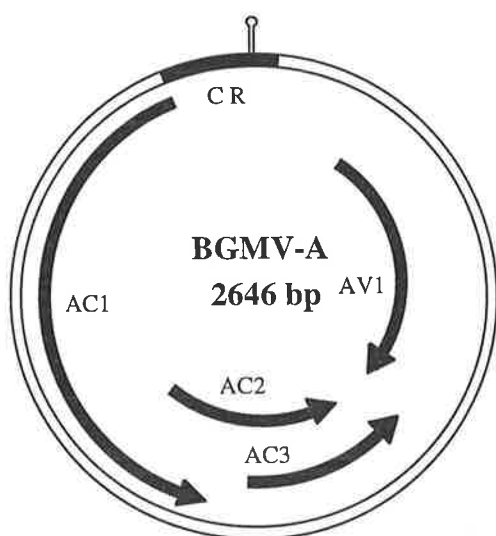
DNA components of the representative members of each geminivirus subgroup are shown. Functional ORFs on both the virion-sense (V) and the complementary-sense (C) DNA strands are displayed by arrows. A and B identify the two DNA components of the bipartite genome of subgroup III viruses. The position of the conserved stem-loop structure ( ), large intergenic region (LIR) and small intergenic region (SIR) of MSV, the intergenic region (IR) of BCTV and TLCV and the common region (CR) of BGMV are marked. Genome organisations are presented to scale using the nucleotide sequence data reported in the GenBank Sequence Database under accession numbers X94330 for MSV (Peterschmitt et al ., 1996), U02311 for BCTV (Stenger, 1994), M10070 for BGMV-DNA A, M10080 for BGMV-DNA B (Howarth et al ., 1985), and S53251 for TLCV (Dry et al ., 1993).

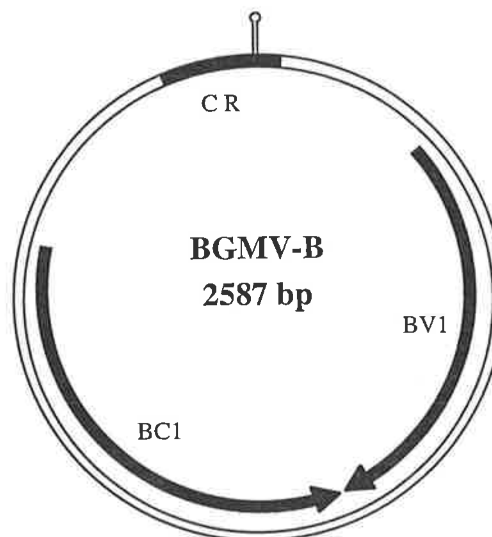


A - Subgroup I

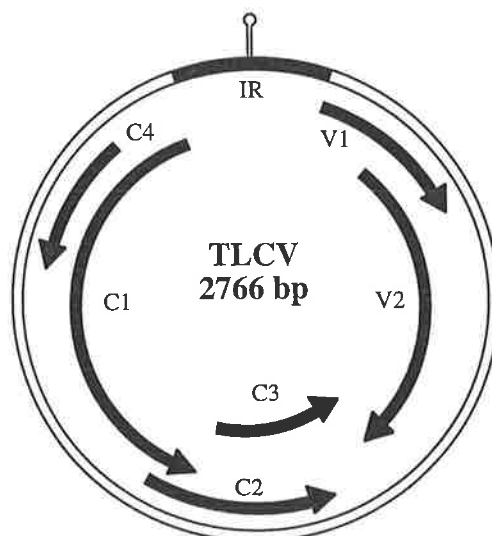


B - Subgroup II





C - Subgroup III, bipartite



D - Subgroup III, monopartite

1.5 Significance of geminiviruses

Geminiviruses have emerged as devastating disease agents in the intensive agricultural regions of the tropics and subtropics throughout the world (Fig. 1.3). Over fifty diseases have been reported in cotton and a range of horticultural crops including tomato, melon, pepper, legumes, watermelon, cassava and squash (Markham *et al.*, 1994).

Fig. 1.3 *Worldwide distribution of geminiviruses*

The prevalence and distribution of these viruses and their vectors have escalated in recent years. The outbreaks of diseases caused by geminiviruses have coincided with the occurrence of new biotypes of the whitefly *B. tabaci*, mostly the 'B' biotype or silverleaf whitefly, the vector of subgroup III geminiviruses. The B biotype feeds on a broad range of plants, possibly in excess of 600 species (Markham *et al.*, 1994). This wide ranging feeding behaviour allows the whitefly to mobilise geminiviruses occurring in natural plant populations into damaging situations in crop plants. Since the new whitefly

biotypes can feed on so many different plant species, there in an opportunity for uninterrupted whitefly regenerations, together with the maintenance of virus reservoirs, throughout the year, resulting in huge populations (De Barro, 1995; Markham *et al.*, 1994).

1.6 Geminiviruses causing leaf curl disease in tomato

Many geminiviruses infect tomato, and leaf curl is one of the most serious diseases they induce. The level of damage ranges from severe to complete crop loss (Makkouk *et al.,* 1979; Polston *et al.*, 1994). Leaf curl disease of tomato is caused by a number of whitefly-transmitted geminiviruses (subgroup III), all of which share the name tomato (yellow) leaf curl virus [T(Y)LCV]. These viruses have been classified as Old World geminiviruses (Fig 1.1). However, in 1994, TYLCV was also identified for the first time in the New World, in the Dominican Republic (Nakhla *et al.*, 1994; Polston *et al.*, 1994).

The initial identification of geminiviruses infecting tomato as T(Y)LCV has been based essentially on the disease syndrome rather than on virus properties. However, more recent molecular characterisation of these viruses has revealed that T(Y)LCV isolated from different countries or geographical regions cannot be considered as different strains of the same virus. For example, certain isolates of T(Y)LCV are more similar at the nucleotide level to geminiviruses infecting other hosts than to other tomato-infecting geminiviruses (Hong *et al.,* 1993; Kheyr-Pour *et al.*, 1991; Rochester *et al.*, 1994; refer also to Fig. 1.1). The molecular differences between these viruses are substantial, despite their similar biology.

Tomato (yellow) leaf curl geminiviruses have been found to possess either

monopartite or bipartite DNA genomes. The names TYLCV and TLCV for these geminiviruses are independent of genomic components. Nucleotide sequence comparisons have been reported amongst T(Y)LC isolates from Australia (TLCV; Dry *et al.*, 1993), Israel (TYLCV-Is; Antignus and Cohen, 1994; Navot *et al.*, 1991), Italy (Sardinia, TYLCV-Sr; Kheyr-Pour *et al.*, 1991) and Spain (Noris *et al.*, 1994), all of which have a single genomic component. In contrast, TLCV isolates from India (TLCV-In; Padidam *et al.*, 1995b) and Thailand (TYLCV-Th; Rochester *et al.*, 1994) have two genomic components. However, only the A component of TYLCV-Th is required to induce disease symptoms in tomato and *N. benthamiana* (Rochester *et al.*, 1990).

1.6.1 Geographical distribution

Originally tomato (yellow) leaf curl geminivirus was widely reported as an economic problem in the Mediterranean region (Al-Musa, 1982; Cherif and Russo, 1983; Cohen and Nitzany, 1966; Czosnek *et al.*, 1990; Makkouk, 1978; Makkouk and Laterrot, 1983; Makkouk *et al.*, 1979; Mazyad *et al.*, 1979). Since these early reports, T(Y)LC viruses have spread rapidly throughout the Middle East (Hajimorad *et al.*, 1996; Mansour and Al-Musa, 1992) and have also been reported in Southeast Asia (Rochester *et al.*, 1990), North and Central Africa (Harrison *et al.*, 1991), the Americas (Brown and Bird, 1992; Brown and Nelson, 1988; Harrison *et al.*, 1991; McGlashan *et al.*, 1994; Nakhla *et al.*, 1994; Polston *et al.*, 1994), and Europe (Kheyr-Pour *et al.*, 1991; Macintosh *et al.*, 1992; Noris *et al.*, 1994) as well as Australia (Buchen-Osmond, 1988).

A recent worldwide survey of tomato yellow leaf curl viruses, using hybridisation tests with two DNA probes derived from a cloned isolate of TYLCV-Is, indicated that the tomato-infecting geminiviruses fall into three main geographical clusters

representing viruses from: 1) the Mediterranean/Middle East/African region; 2) India, the Far East and Australia and 3) the Americas (Czosnek and Laterrot, 1997). Within the first cluster, two sub-clusters of viruses from the western Mediterranean or from the Middle East/Caribbean Islands were distinguished. It has been suggested that the outbreak of TYLCV in the Caribbean Islands was due to an import from the Middle East, causing the rapid spread of the virus to the three major islands: Cuba, Jamaica and Dominican Republic - Haiti (Czosnek and Laterrot, 1997).

Tomato leaf curl disease was first recorded in Australia in 1970 by Aldrick near Darwin in the Northern Territory (NT), where indigenous populations of *B. tabaci* (the vector of TLCV) occur (Buchen-Osmond, 1988). The disease has appeared each year subsequently in NT coastal areas (Condé and Connelly, personal communication, 1996). The silverleaf whitefly (B-biotype of *B. tabaci*) has also been recently introduced into Australia (Gunning *et al.*, 1995) and spread to most agricultural areas including NSW, Queensland, NT and Western Australia. This is posing a serious risk of TLCV spread in tomato crops and is likely to extend the region of TLCV infection.

1.6.2 Transmission

All subgroup III geminiviruses [including T(Y)LCV] are specifically transmitted by the whitefly *B. tabaci* Gennadius (Homoptera: Aleyrodidae) in a persistent (circulative) manner. The recent spread of subgroup III geminiviruses has paralleled the worldwide expansion of the B biotype of *B. tabaci*, which has a wider host range, greater fecundity and more aggressive feeding habit than the endemic biotypes (Bedford *et al.*, 1994; Czosnek and Laterrot, 1997; De Barro, 1995)

B. tabaci is commonly known as the cotton, sweet potato or tobacco whitefly. It

is a phloem feeder and is therefore uniquely adapted to the transmission of subgroup III geminiviruses, which are largely confined to phloem-associated tissues (Dhar and Singh, 1995). Different virus-vector combinations vary in the required acquisition time, latent period and persistence in the vector, and inoculation time. For example for the Israel isolate of TYLCV minimum acquisition and inoculation feeding periods were reported to be 15-30 minutes. The latent period in the vector is more than 20 hours. The virus is retained by the vector up to 20 days but not throughout the life span of the whitefly (Cohen and Nitzany, 1966). The virus can be acquired by larval as well as adult stages of the insect (Zeidan and Czosnek, 1991).

The majority of subgroup III geminiviruses including T(Y)LCV are not mechanically transmissible, however some members including TGMV (Blair *et al.*, 1995), ACMV (Briddon *et al.*, 1993), TMoV (Gilbertson *et al.*, 1993b), BGMV (Morales and Niessen, 1988), and ToYMV (Piven *et al.,* 1995) are transmitted by mechanical inoculation. To date no cases of soil or seed transmission of T(Y)LCV have been reported (Cohen and Nitzany, 1966; Makkouk *et al.,* 1979; Pico *et al.,* 1996).

1.6.3 Host range

The host range of tomato (yellow) leaf curl geminiviruses is narrow and is usually confined to the members of the Solanaceae. In nature, these viruses mainly infect tomato, but tobacco (*Nicotiana tabacum*), eggplant (*Solanum melongena*), datura (*Datura stramonium*) and zinnia (*Zinnia elegans*) have been reported as natural hosts (Conde and Connelly, 1994). Weeds, including those belonging to the species *Euphorbia, Acanthospermum, Ageratum* and *Parthenium*, are considered important reservoirs of TLCV-In (Saikia and Muniyappa, 1989). Cohen and Nitzany (1966) studied the

experimental host range of an isolate of TYLCV-Is in 40 species and varieties of plants in nine families using whitefly transmission tests. *Lycopersicon esculentum, L. hirsutum* and datura were found to be susceptible to the virus and developed clear symptoms. In certain species belonging to Solanaceae *(L. peruvianum, L. pimpinellifolium, Nicotiana glutinosa, and N. tabacum* cv. Samsun*)*, Compositae *(Malva niaensis*) and Leguminosae (*Lens culinaris and Phaseolus vulgaris* cv. Bulagarit), no symptoms were observed, although systemic infection was established by transmission tests. Other species tested were apparently immune.

1.6.4 Symptoms of T(Y)LCV infection

T(Y)LCV infections cause stunting, chlorotic mottling, curling, chlorosis and thickening of leaves and low fruit yield (Pico *et al.,* 1996). The type and severity of symptoms vary according to the virus isolate, host genetic background, environmental conditions, growth stage and physiological conditions of the tomato plant at the time of infection (Pico *et al.,* 1996). Condé and Connelly (1994) described the symptoms of TLCV on tomato plants as follows:

> Affected plants exhibit a greatly reduced growth rate and become stunted. Leaflets are rolled upward and inwards while the leaves are often bent downward (epinasty). Leaves are stiff, thicker than normal and of a leathery texture and often have a purple tinge to the venation on the undersurface. The newly produced leaves are paler in colour (chlorotic) than those on healthy plants. Fruit, if produced on affected plants is smaller than normal, dry in texture and unsalable.

Typical symptoms of TLCV infection in the field are shown in Fig. 1.4.

Fig.1.4 A tomato field in Darwin, Northern Territory, Australia showing TLCV-infected tomato plants (left) along with an apparently healthy plant (right) (M. A. Rezaian, unpublished).

1.6.5 Tomato leaf curl virus from Australia and its relationship with other geminiviruses

Plants infected with TLCV contain characteristic geminate virus particles (Thomas *et al.*, 1986). TLCV has a monopartite genome composed of 2766 nucleotides (Dry *et al.*, 1993). The gene organisation of TLCV is shown in Fig. 1.2D.

The similarity between the sequences of TLCV viral DNA and its ORFs and the equivalent viral DNA and ORFs from a range of other geminiviruses is shown in Table 1.4 (Dry *et al.*, 1993). Alignment of complete nucleotide sequences of TLCV with those of other geminiviruses has shown that TLCV DNA is most similar to the DNA of two

isolates of TYLCV, i.e. TYLCV-Is (75.9% similarity) and TYLCV-Sr (73.2 %) and the DNA A component of ACMV (71.9 %) (Dry *et al.*, 1993). Based on amino acid sequence homology (Table 1.4), TLCV appears to be more closely related to the A component of the bipartite geminivirus ACMV than to TYLCV-Sr. Thus, Dry *et al.* (1993) concluded that sequence comparison alone may not be a sufficient criterion for establishing the relationship between these viruses.

Table 1.4 *Nucleotide and amino acid sequence similarity between TLCV and selected geminiviruses*

Virus[a]	Genomic sequence accession number	Total nucleotide similarity[a] (%)	Amino acid identity[b] (%) ORFs					
			V1	V2	C1	C2	C3	C4
AbMVA	X15983	69.1	-	73	68	47	52	60
ACMVA	J02058	71.9	63	75	78	62	63	48
BGMVA	M10070	66.6	-	71	64	45	51	52
BCTV	X04144	61.9	15	17	61	20	59	55
MSV	X01633	50.1	14	10	25	-	-	-
PYMVA	D00940	69.5	-	71	68	52	52	[c]n.a.
TGMVA	K02030	69.9	-	74	69	58	55	51
TYDV	M81103	51.9	10	11	25	-	-	-
TYLCV-Is	X15656	75.9	62	75	83	61	68	50
TYLCV-Sr	X61153	73.2	58	73	79	56	59	63
WDV	X02869	49.2	17	6	24	-	-	-

[a]AbMVA, AbMV DNA A (Frischmuth *et al.*, 1990); ACMVA, ACMV DNA A (Stanley and Gay, 1983); BGMVA, BGMV DNA A (Howarth *et al.*, 1985); BCTV (Stanley *et al.*, 1986); MSV (Mullineaux *et al.*, 1984); PYMVA, PYMV DNA A (Coutts *et al.*, 1991); TGMVA, TGMV DNA A (Hamilton *et al.*, 1984); TYDV (Morris *et al.*, 1992); TYLCV-Is (Navot *et al.*, 1991); TYLCV-Sr (Kheyr-Pour *et al.*, 1991); WDV (MacDowell *et al.*, 1985).

[b] Sequence alignment was carried out using NALIGN and PNALIGN from the PC/GENE sequence analysis software package (IntelliGenetics Inc., Mountain View, CA). The coat protein ORFs of ACMVA, AMBVA, BGMVA, PYMVA, TGMVA, and TYLCV-Is have been defined as V2 for this comparison. No values are given where comparable ORFs do not exist (Dry *et al.*, 1993).

[c]n.a., Not available

1.7 Geminiviral gene functions

The function of different ORF products of geminiviruses have been studied extensively using viral mutants in whole plants and in *in vitro* assays including leaf discs or protoplasts. Plant transformation has also been used to confirm geminiviral gene functions suggested by the mutational analysis. Recent studies using purified translated products of geminiviral ORFs have also led to a better understanding of geminiviral gene functions. Here, these functions will be considered with an emphasis on subgroup III viruses and where possible on T(Y)LCV.

1.7.1 V1 ORF

The V1 ORF of monopartite subgroup III geminiviruses is involved in viral ssDNA accumulation and symptom development (Rigden *et al.*, 1993; Wartig *et al.*, 1997). However, the role of this ORF in systemic viral movement in the plant is less clear. Rigden *et al.* (1993) reported that disruption of the V1 ORF of TLCV did not affect the synthesis of viral dsDNA but led to systemic and symptomless infection with a reduced titre of all viral DNA forms in inoculated tomato and datura plants. They concluded that the V1 ORF is not required for systemic viral movement in plants. In contrast to TLCV, a disrupted ORF V1 of TYLCV-Sr abolished systemic infection in tomato plants (Wartig *et al.*, 1997). Warting *et al.* (1997) concluded that this ORF is required for a successful infection process in this host.

A functional ORF analogue of V1 is present in the subgroup II BCTV, where it has been shown to be involved in the synthesis or accumulation of ssDNA. It is required for efficient systemic infection and symptom development (Stanley *et al.*, 1992). A symptomless and systemic infection associated with low levels of virus was observed in

N. benthamiana plants infected with a mutant construct of BCTV with a disruption in this ORF. However, this BCTV mutant was non-infectious in *Beta vulgaris*, suggesting that this gene product has a functional role in virus spread in the natural host *Beta vulgaris* but is not necessary for infectivity in permissive hosts such as *N. benthamiana* (Stanley *et al.*, 1992).

The V1 ORF of monopartite subgroup I geminiviruses is required for infectivity, and is probably involved in systemic spread and symptom development (Boulton *et al.*, 1991; Boulton *et al.*, 1989; Lazarowitz *et al.*, 1989). In WDV, the V1 ORF has been implicated in cell-to-cell spread function (Dekker *et al.*, 1991). Since ORF V1 overlaps a possible regulatory element of the coat protein gene, it has been argued that mutation in this ORF may affect infectivity by altering expression of the coat protein (Boulton *et al.*, 1989; Lazarowitz *et al.*, 1989). Such an indirect effect is unlikely, however, because some of the V1 ORF mutants produce viral particles in maize protoplasts, suggesting that the production of coat protein is not dependent upon the product of V1 ORF (Boulton *et al.*, 1993). On the basis of this observation, Boulton *et al.* (1993) concluded that the V1 protein of MSV and similar viruses in subgroup I may be necessary for systemic infection of the virus, but not in the sense of a "conventional" movement protein.

1.7.2 V2 (AV1) ORF

The function of the monopartite T(Y)LCV ORF V2, which encodes the viral coat protein, is common to other monopartite geminiviruses belonging to subgroups I and II (Boulton *et al.*, 1989; Briddon *et al.*, 1989; Dry *et al.*, 1993; Morris-Krsinich *et al.*, 1985; Rigden *et al.*, 1993). The coat protein is essential for systemic spread and

infectivity. Deletion and insertion mutations in the coat protein genes of TLCV, TYLCV-Sr, BCTV, MSV, and WDV have been found to destroy viral spread and infectivity in plants (Boulton *et al.*, 1989; Briddon *et al.*, 1989; Lazarowitz *et al.*, 1989; Rigden *et al.*, 1993; Wartig *et al.*, 1997; Woolston *et al.*, 1989). The coat protein mutant constructs have been found to replicate viral DNA at the site of inoculation or in protoplast-derived cells, although ssDNA occurs at low levels (Rigden *et al.*, 1993; Wartig *et al.*, 1997; Woolston *et al.*, 1989), similar to the situation for coat protein mutants of the bipartite geminiviruses (see below).

The function of the coat protein gene has also been studied in the bipartite TLCV-In and TYLCV-Th (Padidam *et al.*, 1995b; Rochester *et al.*, 1994). It has been shown to be common with other bipartite viruses such as TGMV and ACMV. In contrast to the monopartite T(Y)LC geminiviruses, the coat protein of bipartite geminiviruses (Kallender *et al.*, 1988; Padidam *et al.*, 1995b; Townsend *et al.*, 1985) is not essential for systemic spread and symptom development. Coat protein mutants of TLCV-In, TYLCV-Th, TGMV and ACMV have been found to be infectious (Brough *et al.*, 1988; Gardiner *et al.*, 1988; Padidam *et al.*, 1995b; Rochester *et al.*, 1994; Stanley and Townsend, 1986). However, coat protein mutants of some of these viruses produce delayed and attenuated disease symptoms (Brough *et al.*, 1988; Gardiner *et al.*, 1988; Rochester *et al.*, 1994), suggesting a role in systemic spread (Gardiner *et al.*, 1988) or in enhancing disease progression (Rochester *et al.*, 1994). Furthermore, elimination of the coat protein gene has been shown to result in a reduction in the level of viral ssDNA (Padidam *et al.*, 1995b; Rochester *et al.*, 1994; Stanley and Townsend, 1986), presumably due to nuclease degradation (Stanley and Townsend, 1986).

Generally, the geminiviral coat protein is thought to determine vector specificity. This idea is supported by the strong serological relationships and sequence homology between the coat proteins of the subgroup III viruses, which are all transmitted by a single *B. tabaci* whitefly species (Roberts *et al.,* 1984). In contrast, the coat proteins of the leafhopper-transmitted geminiviruses BCTV, MSV and WDV, which have different vector species, show much less sequence homology and have a distant serological relationship (Roberts *et al.,* 1984). Further evidence for the role of coat protein in vector specificity has been obtained by replacing the coat protein-coding region of ACMV with the coat protein gene from BCTV. The chimeric virus was transmitted by the BCTV leafhopper vector, *Circulifer tenellus*, but unmodified ACMV was not (Briddon *et al.*, 1990).

1.7.3 C1 (AC1) ORF

The C1 (AC1) ORF of the subgroup III geminiviruses encodes a protein designated the replication-associated protein (Rep). Mutational analysis of TLCV and several other subgroup III geminiviruses has shown that Rep is the only viral gene product absolutely required for viral replication (Brough *et al.*, 1988; Dry *et al.*, 1997; Elmer *et al.*, 1988; Etessami *et al.*, 1991; Hanley-Bowdoin *et al.,* 1990; Rigden *et al.*, 1996).

The expression of AC1 ORF in transgenic plants is sufficient for ss and dsDNA replication of TGMV in the presence of host proteins (Hanley-Bowdoin *et al.,* 1990; Hayes and Buck, 1989). The C1 ORF of monopartite subgroup II BCTV share significant homology with C1 (AC1) ORF of subgroup III geminiviruses and has a similar function (Briddon *et al.*, 1989).

Analogous to the requirement for the *rep* gene in the replication of the subgroup III viruses, the C1 and C2 ORFs of subgroup I geminiviruses encode an homologous product essential for DNA replication (see Fig. 1.5) (Lazarowitz *et al.*, 1989; Schalk *et al.*, 1989). In this case, Rep is expressed via a spliced mRNA (Accotto *et al.*, 1989; Schalk *et al.*, 1989).

1.7.3.1 Rep is a multifunctional protein.

The C1 ORF is the most extensively studied geminivirus gene. Because of the close functional similarity of the C1 gene in various geminiviruses, a clear picture has emerged relating to its role from various studies. Rep has been expressed and purified *in vitro* in both monopartite and bipartite subgroup III geminiviruses (Fontes *et al.*, 1992; Laufs *et al.*, 1995c; Orozco *et al.*, 1997; Thommes *et al.*, 1993) and it has been shown to be a multifunctional protein.

Studies with TYLCV and TGMV have shown that Rep binds specifically to sequence motifs of viral DNA located just upstream of the hairpin structure in the intergenic region (Fontes *et al.*, 1992; Fontes *et al.*, 1994a; Jupin *et al.*, 1995; Thommes *et al.*, 1993). Using purified Rep protein of TYLCV *in vitro*, it has been shown that the Rep possesses nicking and joining activity. It cleaves and ligates the viral DNA strands in the conserved loop sequence of the hairpin (Heyraud-Nitschke *et al.*, 1995; Laufs *et al.*, 1995c).

Both ATPase and GTPase activities have been demonstrated for TYLCV Rep and are thought to be required for viral DNA replication (Desbiez *et al.*, 1995). Rep is also involved in activation as well as repression of its own transcription (Eagle *et al.*, 1994; Hofer *et al.*, 1992; Sunter *et al.*, 1993).

1.7.3.2 Functional domains of the Rep protein

Rep has no homology to any known DNA polymerases (Laufs *et al.*, 1995c) but contains motifs conserved in proteins catalysing the initiation of DNA replication of some ssDNA prokaryotic plasmids (Gorbalenya *et al.,* 1990; Ilyina and Koonin, 1992; Koonin and Ilyina, 1992). The locations of four motifs conserved among the Reps of geminiviruses are presented in Fig. 1.5.

Recent experiments have begun to identify the function of Rep domains in geminiviruses. By expressing the different domains of the TYLCV-Sr Rep protein in *E. coli,* Heyraud-Nitschke *et al.* (1995) demonstrated that the cleavage and joining activities of the geminivirus Rep mapped to the first 211 amino acids of this protein. This region contains three amino acid motifs (Fig. 1.5). The function of motif I remains unknown. Motif II contains two conserved histidine (H) residues and has been suggested to be involved in metal ion coordination required for the activity of Rep (Ilyina and Koonin, 1992; Koonin and Ilyina, 1992). Motif III contains a conserved tyrosine (Y) which initiates DNA cleavage and forms a covalent link with nicked DNA (Koonin and Ilyina, 1992; Laufs *et al.*, 1995b). A lysine (K) residue located 3 residues downstream of the tyrosine residue in motif III is invariant in all whitefly-transmitted geminiviruses and is essential for viral replication (Hoogstraten *et al.,* 1996).

Motif IV, localised in the C-terminus of geminiviral Rep (Fig. 1.5), represents a nucleoside triphosphate binding site (P-loop) (Desbiez *et al.*, 1995; Gorbalenya *et al.,* 1990) which has been shown to be essential for the ATPase activity of Rep protein *in vitro* and for geminiviral replication *in vivo* (Desbiez *et al.*, 1995; Hanson *et al.*, 1995).

```
                1                  I                          50
TLCV        ..MTRPKSFRINAKNYFLTYPKCSLTKEEALSQLNNLETPTSKKYIKVCR
TYLCV-Sr    ..MPRSGRFSIKAKNYFLTYPKCDLTKENALSQITNLQTPTNKLFIKICR
BGMV        ..MPPPQRFRVQSKNYFLTYPRCTIPKEEALSQLQKIHTTTNKKFIKVCE
TGMV        .MPSHPKRFQINAKNYFLTYPQCSLSKEESLSQLQALNTPINKKFIKICR
BCTV        ..MPPTKRFRIQAKNIFLTYPQCSLSKEEALEQIQRIQLSSNKKYIKIAR
 WDV        MASSSAPRFRVYSKYLFLTYPECTLEPQYALDSLRTLLNKYEPLYIAAVR

            51     II                                        100
TLCV        ELHENGEPHLHVLIQFEGKFQCKNQRFFDL.VSPTRSAHFHPNIQGAKSS
TYLCV-Sr    ELHENGEPHLHILIQFEGKYNCTNQRFFDL.VSPTRSAHFHPNIQGAKSS
BGMV        ERHDNGEPHLHALIQFEGKFICTNKRLFDL.VSTTRSAHFHPNIQGAKSS
TGMV        ELHEDGQPHLHVLIQFEGKYCCQNQRFFDL.VSPTRSAHFHPNIQRAKSS
BCTV        ELHEDGQPHLHVLLQLEGKVQITNIRLFDL.VSPTRSAHFHPNIQRAKSS
 WDV        ELHEDGSPHLHVLVQNKLRASITNPNALNLRMDTSPFSIFHPNIQAAKDC

            101  III                                         150
TLCV        SDVKSYLEKDGD....TLEWGEFQIDGRSARGGQQSANDAYAQALNTGSK
TYLCV-Sr    SDVKSYIDKDGD....VLEWGTFQIDGRSARGGQQTANDAYAKAINAGSK
BGMV        SDVKEYIDKDGV....TIEWGQFQVDGRSARGGQQSANDSYAKALNADSI
TGMV        SDVKTYIDKDGD....TLVWGEFQVDGRSARGGCQTSNDAAAEALNASSK
BCTV        SDVKSYVDKDGD....TIEWGEFQIDGRSARGGQQTANDSYAKALNATSL
 WDV        NQVRDYITKEVDSDVNTAEWGTFVAVSTPGRKDRDADMKQIIES..SSSR

            151                                              200
TLCV        SEALNVLRELAPKDYVLQFHNLNSNLDRIFTPPLEVYVSPFLSSSFDRVP
TYLCV-Sr    SQALDVIKELAPRDYVLHFHNINSNLDKVFQVPPAPYVSPFLSSSFDQVP
BGMV        ESALTILKEEQPKDYVLQNHNIRSNLERIFFKVPEPWVPPFPLSSFVNIP
TGMV        EEALQIIREKIPEKYLFQFHNLNSNLDRIFDKTPEPWLPPFHVSSFTNVP
BCTV        DQALQILKEEQPKDYFLQHHNLLNNAQKIFQRPPDPWTPLFPLSSFTNVP
 WDV        EEFLSMVCNRFPFEWSIRLKDFEYTARHLFPDPVATYTPEFPTESLI.CH

            201                                IV            250
TLCV        EELEEWVAEN.VKDAAARPLRPISIVIEGESRTGKTVWARSLGPHNYLCG
TYLCV-Sr    DELEHWVSEN.VMDAAARPWRPVSIVIEGDSRTGKTTWARSLGPHNYLCG
BGMV        VVMQDWVDDYFGRGSAARPERPISIIVEGDSRTGKTMWARALGPHNYLSG
TGMV        DEMRQWAENYFGKSSAARPERPISIIIEGDSRTGKTMWARSLGPHNYLSG
BCTV        EEMQEWADAYFGVDAAARPLRYNSIIVEGDSRTGKTMWARSLGAHNYITG
 WDV        ETIESWKNEHLYS***ESPGRHKSIYICGPTRTGKTSWARSLGTHNYYNS
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Fig. 1.5 *Alignment of the amino acid sequence of the Rep (approximately 2/3 of protein) of TLCV, TYLCV-Sr, BGMV, TGMV, , BCTV and WDV. Four conserved motifs (I to IV) are indicated by boxes. Dots indicate residues where gaps have been inserted to allow alignment of sequences. Stars indicate the splice junction of C1 and C2 ORFs of WDV to produce the functional Rep protein (refer to Schalk et al ., 1989).*

Using a series of purified truncated TGMV Rep, Orozco *et al*. (1997) localised the DNA binding domain of this virus in the first 181 amino acids of Rep. In these experiments deletion of the first 29 amino acids of AC1 abolished DNA binding. These observations show that DNA binding domains are located in the N-terminus of Rep. Orozco *et al*. (1997) also demonstrated that the AC1 contains an oligomerization domain which is located between amino acids 121 and 181 of TGMV Rep.

1.7.4 C2 (AC2) ORF

The C2 gene product of monopartite subgroup III geminiviruses is required for systemic infection. A C2 mutant of TLCV retained the capability of autonomous replication in tobacco leaf strips (Dry *et al*., 1997), but was not infectious when agroinoculated in tomato and datura plants (I. B. Dry, personal communication, 1997). Agroinoculation of tobacco leaf strips with this mutant resulted in the production of the replicative DNA forms, with a reduced level of ssDNA relative to the dsDNA forms when compared to the wild type infection. This is consistent with the suggestion that the C2 gene product transactivates virion-sense genes and is required for coat protein synthesis (Dry *et al*., 1997), as shown for bipartite geminiviruses (see below). The C2 gene product of TYLCV-Sr has also been shown to be required for infectivity in tomato plants but not in *N. benthamiana*, where systemic infection with attenuated symptoms has been observed (Wartig *et al*., 1997).

The AC2 ORF of bipartite geminiviruses plays an important role in viral movement. Mutation in the AC2 ORF of TGMV prevents systemic viral movement in plants and results in reduced amounts of ssDNA in transient assays (Sunter and Bisaro, 1991; Sunter and Bisaro, 1992). The protein encoded by this gene is a transactivator of

the AV1 (coat protein) and BV1 genes (Sunter and Bisaro, 1991; Sunter and Bisaro, 1992) and its role in virus movement appears to be through this regulatory function. AC2 has also been shown to regulate the expression of BC1 in ACMV (Haley *et al.*, 1992). It seems that the function of AC2 gene of bipartite geminiviruses is not virus specific, as an AC2 mutant of PYMV can be complemented by DNA A of TGMV to restore infectivity in *N. benthamiana* (Sung and Coutts, 1995).

In contrast to the AC2 ORF of bipartite geminiviruses, the C2 ORF of BCTV is not required for infectivity. Mutational experiments indicated that BCTV C2 mutants were infectious and that the product of this gene was not needed for V2 (coat protein) expression (Hormuzdi and Bisaro, 1995; Stanley *et al.*, 1992). In these experiments the BCTV C2 could be mutated without affecting the accumulation of viral ss and dsDNA forms leading to the conclusion that the C2 protein was not needed for expression of the V1 gene, which has been shown to be required for maintaining ratios of ss and dsDNA (Hormuzdi and Bisaro, 1993; Stanley *et al.*, 1992).

The ability of the TYLCV C2 protein to bind to the viral DNA has been investigated by Noris *et al.* (1996) using purified TYLCV C2 protein in Southwestern blotting and electrophoretic mobility shift assays. The C2 protein appears to bind to DNA in a sequence-non-specific manner (Noris *et al.*, 1996). Similar non-specific binding has also been observed with the AC2 protein of PYMV (Sung and Coutts, 1996).

1.7.5 C3 (AC3) ORF

The role of the T(Y)LCV C3 gene has not been extensively studied. However, mutational studies conducted on TLCV (I. B. Dry, personal communication, 1997)

suggest that the C3 gene of this virus performs a similar function to the analogous gene in bipartite subgroup III viruses. A C3 mutant of TLCV, like AC3 mutants of TGMV and ACMV (Brough *et al.*, 1988; Elmer *et al.*, 1988; Etessami *et al.*, 1991; Morris *et al.*, 1991), was found to be infectious, although agroinoculated plants accumulated lower levels of viral DNA than plants agroinoculated with a wild type construct and produced delayed and attenuated symptoms. The AC3 ORF of the bipartite subgroup III geminiviruses is not essential for viral replication; however it appears to influence the efficiency of viral DNA synthesis in enhancing viral DNA replication levels (Sunter *et al.*, 1990). It is possible that the AC3 protein transactivates the expression of AC1 (Sunter *et al.*, 1990); however, how this is achieved is unknown. Sung and Coutts (1995) demonstrated that the TGMV DNA A complemented a PYMV AC3 mutant and restored the reduced DNA phenotype, showing that the function of AC3 ORF, like AC2, is not virus specific.

Mutational analysis indicates that the BCTV C3 gene is functionally homologous to the bipartite geminivirus AC3 gene (Hormuzdi and Bisaro, 1995; Stanley *et al.*, 1992). The BCTV C3 protein has been shown to enhance the replication of a TGMV AC3 mutant back to essentially wild type levels, and vice versa (Hormuzdi and Bisaro, 1995).

1.7.6 C4 ORF

ORF C4 of monopartite subgroup III geminiviruses, which is entirely contained within the C1 ORF, appears to be necessary for eliciting disease symptoms, but is not necessary for viral replication. Jupin *et al.* (1994) have suggested that the C4 ORF of TYLCV is involved in viral systemic movement, because when disrupted, the virus was

unable to move systemically and infected tomato plants remained asymptomatic. In contrast, mutational analysis of the C4 ORF of TLCV demonstrated that the C4 protein is involved in symptom severity, but not in systemic spread of the virus through the plant (Rigden *et al.*, 1994). Krake *et al.* (1998) have recently demonstrated that the expression of the TLCV C4 ORF produces virus-like symptoms in tobacco and tomato plants, supporting the suggestion that the C4 protein is involved in the development of disease symptoms during viral infection.

Similar to the case of TLCV, it has been shown that the C4 ORF of BCTV, a subgroup II geminivirus, is a determinant of pathogenesis and plays a significant role in the development of disease symptoms. However, the C4 ORF of BCTV has host-specific effects on infectivity and symptomatology (Stanley and Latham, 1992). Expression of the C4 ORF of BCTV in transgenic *N. benthamiana* plants resulted in abnormal plant development and the production of tumorigenic growths, showing a role for this protein in the regulation of host cell division (Latham *et al.*, 1997).

In contrast to the C4 ORF of monopartite subgroup III geminiviruses, the analogous AC4 ORF of bipartite geminiviruses has not been shown to have a role in viral movement or symptom development (Elmer *et al.*, 1988; Etessami *et al.*, 1991; Hoogstraten *et al.*, 1996; Pooma and Petty, 1996).

1.7.7 BC1 and BV1 ORFs

The BC1 and BV1 ORFs, the only two genes contained on the B component, are present in the bipartite geminiviruses including certain tomato-infecting viruses. The roles of these genes in the bipartite TLCV-In (Padidam *et al.*, 1995b) and TYLCV-Th (Rochester *et al.*, 1994) have not been studied. However, given the close similarity

between the genome organisation of these two viruses and those of other bipartite subgroup III geminiviruses, it seems likely that the BC1 and BV1 ORFs in these viruses perform similar functions.

The BC1 and BV1 ORFs are involved in viral movements in the host plant. Mutations of these genes do not affect viral DNA replication, as determined in leaf disc or protoplast assays, but prevent infectivity in the whole plants (Brough *et al.*, 1988; Etessami *et al.*, 1988; Sunter *et al.*, 1990). Alignment of the BV1 and BC1 products of ACMV with those of seven other bipartite geminiviruses (AbMV, BGMV, BDMV, MYMV, SqLCV, PYMV and TGMV) showed several highly conserved regions in these proteins, which have been shown to be essential for systemic spread (Haley *et al.*, 1995).

Mutational analysis has indicated that the BC1 ORF is involved in short distance cell-to-cell spread (Hayes and Buck, 1989; Jeffrey *et al.*, 1996). The localisation of the BC1 protein of ACMV (Von Arnim *et al.*, 1993) and SqLCV (Pascal *et al.*, 1993) to the cell wall and plasma membrane, has led to the suggestion that this protein may alter plasmodesmata, in a similar way to the 30-kDa movement protein of tobacco mosaic virus (Maule, 1991; Wolf *et al.*, 1991). Recently, Ward *et al.* (1997), using immunolocalization experiments, demonstrated that SqLCV BC1 movement protein is associated with endoplasmic reticulum-derived tubules in developing phloem cells. They concluded that SqLCV appeared to recruit the endoplasmic reticulum as a conduit for cell-to-cell movement of the viral genome.

The BV1 ORF has been implicated in both long and short distance movements. BV1 mutants of both ACMV and SqLCV did not permit systemic infection, indicating

that the BV1 ORF is essential for long-distance vascular spread of the virus in the host plants (Pascal *et al*., 1993; Von Arnim *et al.,* 1993). A mutation of this gene in ACMV reduced the level of viral DNA within the inoculated leaf tissue, suggesting an involvement of this gene in local spread (Von Arnim *et al.,* 1993). It has also been shown that the BC1 gene is necessary for local TGMV movement (Jeffrey *et al.,* 1996).

Using functional BGMV proteins expressed in *E. coli* and microinjection into plant cells, it has been suggested that the BV1 and BC1 proteins coordinated the movement of viral DNA across both nuclear and plasmodesmal boundaries, and that movement of ss and dsDNA out of the nucleus was mediated by the BC1 protein (Noueiry *et al.,* 1994). Similarly, by investigating the biochemical properties and cellular locations of SqLCV BC1 and BV1 proteins, it has been suggested that BV1 is bound to ssDNA and is involved in the shuttling of the genome in and/or out of the nucleus, whereas BC1 acts at the plasma membrane/cell wall to facilitate viral movement across cell boundaries (Pascal *et al*., 1994).

1.8 Viral replication strategy

Geminiviruses replicate in the nuclei of infected plant cells via a dsDNA intermediate (Saunders *et al.,* 1991; Stenger *et al*., 1991). Viral DNA synthesis is strictly Rep dependent and requires host DNA polymerase(s) and other plant enzymes that normally are expressed or are active during the S-phase of the cell cycle. The role of Rep in host cell cycle regulation to initiate viral replication may involve a Rep domain capable of binding and hydrolysing ATP/GTP (Laufs *et al*., 1995a). An elevated level of proliferating nuclear antigen, a protein associated with the S-phase of cell cycle, has been detected in differentiated leaf cells infected with TGMV and this may indicate

cell cycle modulation by geminiviruses (Laufs *et al*., 1995a; Nagar *et al*., 1995).

Rep may also be involved in unwinding the origin of replication of viral DNA to facilitate loading of host DNA polymerase complexes in a manner analogous to that of the simian virus 40 large tumor antigen (Goetz *et al*., 1988). Evidence for this view exists in amino acid sequence homology between geminiviral Rep(s) and a family of viral helicases (Gorbalenya and Koonin, 1989).

Based on the information summarised above and the similarities between geminiviral Rep and analogous pro- and eukaryote proteins, the geminivirus genomes seem to employ a rolling circle replication mechanism similar to that used by a class of eubacterial plasmids (Gruss and Ehrlich, 1989) and some bacteriophages (Baas and Jansz, 1988).

Following infection and uncoating of the virion DNA, the ss viral strand is first converted to a ds replicative form (RF), with the synthesis of a complementary-sense strand (Dhar and Rudra, 1995). While the subgroup I viruses contain primer-like molecules (Andersen *et al*., 1988; Donson *et al*., 1987; Donson *et al*., 1984; Hayes *et al*., 1988; Howell, 1984; Morris *et al*., 1992), the priming mechanism of other geminiviral DNAs is not understood. The synthesis of the complementary-sense strand is presumed to be host-directed since deproteinized geminivirus ssDNA is infectious (Dhar and Rudra, 1995). According to the rolling circle model, new viral strands are synthesised progressively (see below), displacing the original viral strand from the RF (Chasan, 1995).

The origin of DNA replication (*ori*) of geminiviruses has been localised in the intergenic region (Fontes *et al.,* 1992; Lazarowitz *et al*., 1992; Revington *et al.,* 1989;

Schneider *et al.,* 1992; Stanley, 1995). The binding of Rep to the intergenic region is a prerequisite for initiation of DNA replication. Rep specifically recognises and interacts with sequences within the left part of the intergenic region, as demonstrated for TGMV (Fontes *et al.,* 1992; Thommes *et al.*, 1993), SqLCV (Lazarowitz *et al.*, 1992), BCTV (Choi and Stenger, 1995) and WDV (Heyraud *et al.*, 1993). The hairpin structure does not contribute to the Rep binding and specificity of origin recognition (Fontes *et al.,* 1992).

Rep initiates rolling circle replication by introducing a nick in the plus strand between bases 7 and 8 within the conserved nonamer motif, ^{1}TAATATT↓^{8}AC. The liberated 3′ hydroxyl at the nucleotide position 7 (T) becomes available to prime plus-strand DNA synthesis, while the 5′ end of the cleaved strand becomes covalently linked to Rep (Ilyina and Koonin, 1992; Laufs *et al.*, 1995c). When a round of replication is completed, the newly synthesised origin is cleaved at the same position of the nonanucleotide by Rep, and the resulting 3′ hydroxyl of the displaced strand is joined to the 5′ phosphate of the strand linked to Rep, concomitantly releasing a circular ssDNA molecule of genome length (Laufs *et al.*, 1995c). The released ssDNA molecule could then serve as template for complementary strand synthesis or be packaged into a virion, depending on the stage of the infection (Chasan, 1995).

1.9 Scope of this study

This work is part of a broader research project aimed at understanding the biology of TLCV and developing strategies against this important geminivirus. Within this framework the specific objectives of the work described in this thesis include:

1- Characterising viral agents in infected field samples from the northern part of Australia.

2- Expressing and purifying the replication-associated protein encoded by TLCV.

3- Identifying the Rep binding elements in TLCV DNA.

4- Studying cross interaction of Rep and binding elements between different TLCV strains.

5- Characterising TLCV subgenomic DNAs and their production during virus replication.

Chapter Two

General Materials and Methods

2.1 MATERIALS

2.1.1 Solutions

All chemicals used were analytical reagent or molecular biology grade. Solutions were prepared with ultra-pure water and autoclaved where appropriate. The compositions of buffers and growth media are outlined in Table 2.1. Liquid phenol was equilibrated in 100 mM Tris-HCl pH 8.0 (Sambrook *et al.,* 1989).

2.1.2 Synthetic oligodeoxyribonucleotides

Synthetic oligodeoxyribonucleotides were obtained either from the facilities in the Department of Plant Science, University of Adelaide or from Gibco BRL through Life Technologies, Victoria, Australia. The nucleotide sequences of the oligonucleotides used in this study are listed in Table 2.2.

2.1.3 Bacterial strains and plasmid vectors

Escherichia coli strain DH5α, Stratagene (USA) was used for all routine cloning work. *E. coli* strain M15, QIAGEN (Germany) was used as a host for expression of fusion proteins. *Agrobacterium tumefaciens* strain C58 was used for agroinoculation of TLCV constructs.

Routine cloning was carried out using the vector pBluescript SK^+ (Stratagene, USA), a phagemid carrying ampicillin resistance and with promoter sequences for T3 and T7 RNA polymerases flanking the polylinker. A modified pBluescript SK^+ T-tail vector containing 5′-T overhangs at the *Eco* RV site for the direct cloning of PCR

products was prepared as described by Marchuk *et al.* (1990) and supplied by Dr. I. B. Dry (CSIRO Plant Industry, Horticulture Unit, Adelaide, Australia). The binary vector pBin 19 (Bevan, 1984; Frisch *et al.*, 1995) carrying kanamycin resistance was used vector for *A. tumefaciens*-mediated agroinoculation. The vector pQE30, QIAGEN (Germany) was used for expression of TLCV Rep protein.

***Table 2.1** Solutions and their compositions.*

Solutions	Compositions
Sample loading buffers	
Agarose-gel loading buffer (10x):	78% glycerol, 1.2 mg ml^{-1} ribonuclease A, 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol, 1.0 mM EDTA.
Formamide loading buffer	95% formamide, 20 mM EDTA pH 8.0, 0.05% (w/v) bromophenol blue, 0.05% (w/v) xylene cyanol.
SDS-gel-loading buffer	65 mM Tris-HCl pH 6.8, 2% SDS, 5% β-mercaptoethanol, 10% glycerol, 0.002% (w/v) bromophenol blue.
Extraction buffers	
DNA extraction buffer	50 mM Tris-HCl pH 8, 100 mM NaCl, 10 mm EDTA, 1% (w/v) SDS, 1% (v/v) β-mercaptoethanol.
Protein extraction buffer	50 mM NaH_2PO_4 pH 8.0, 300 mM NaCl, 10 mM β-mercaptoethanol.
Other buffers	
Antibody coating buffer (pH 9.6)	1.59 g Na_2CO_3, 2.93 g $NaHCO_3$, 0.2 g NaN_3 in 1000 ml water.
DNA-protein binding buffer	20 mM Hepes pH 7.4, 40 mM KCl, 10% glycerol, 1 mM DTT.
Phosphate-buffered saline (PBS)-Tween 20	8.0 g NaCl, 0.2 g KH_2PO_4, 2.9 g Na_2HPO_4, 0.2 g KCl, 0.5 ml Tween 20 in 1000 ml water.
Polyacrylamide gel elution buffer	0.5 M ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA pH 8.0, 0.1% SDS.
1x SDS PAGE running buffer	25 mM Tris-HCl pH 8.3, 0.1% SDS, 190 mM glycine
1x SSC	150 mM NaCl, 15 mM trisodium citrate.
STET buffer	8% sucrose, 5% Triton X-100, 50 mM EDTA (pH 8.0), 50 mM Tris-HCl pH 8.0.
TBE (electrophoresis running buffer)	89 mM Tris-borate pH 8.3, 2 mM EDTA.
TE buffer	10 mM Tris-HCl pH 8.0, 0.1 mM EDTA.
Growth media	
Leaf strip culture medium	0.43% MS salts, 3% sucrose. 0.01% Gamborg vitamins. To prepare solid medium, 0.7% bacto-agar was added.
LB broth	1% (w/v) bacto-tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl, pH 7.0. To prepare solid medium, 1.2% bacto-agar was added.

***Table 2.2** Oligonucleotide primers used in this study*

Primers	Virus or viroid*	Size (nt)	Nucleotide positions[a]	Sequences from 5′ to 3′[b]	Underlined restriction site
P1[v]	T-type	18	1-18	TAATATTACCGGATGGCC	
P169[c]	T-type	20	150-169	CGTGGACTAAAGGATCCCAC	*Bam* HI
P272[v]	T-type	25	272-296	TCAGGGATTGCATTGGGGTTGTTAG	
P1527[v]	T-type	27	1527-1544	gcgaagcttTCAATTCTCTTCCTCCGG	*Hind* III
P1583[v]	T-D1	22	1583-1602	catGGTACCTGAGTACAACGGC	*Kpn* I
P1588[c]	T-D1	23	1569-1588	gacGGTACCTATCAAGGTCCAAC	*Kpn* I
P1626[c]	T-type	23	1604-1626	TGCAGAATTCATCACCCTCAACG	
P2025[v]	T-type	26	2025-2048	agCTCGAGTTCTTCTGGAACTCGATC	*Xho* I
P2030[c]	T-type	27	2006-2030	ttCTCGAGGAGTGGGTCGCCGAGAATG	*Xho* I
P2042[c]	T-type	26	2017-2042	GTTCCAGAAGAACTCGAGGAGTGGGT	
P2326[V]	T-type	28	2326-2351	tgGAGCTCTTAGCTCCCTGAATGTTCGG	*Sac* I
P2331[C]	T-type	24	2310-2331	ttGAGCTCGTCAGACGTCAAGTCC	*Sac* I
P2339[v]	T-type	15	2339-2353	CCTGAATGTTCGGAT	
P2386[c]	T-type	25	2362-2386	ACCTGGTCTCCCCCACCAGGTCAGC	
P2593[v]	T-D1	20	2593-2612	TAAAACGCTTAGGGGGTGCC	
P2594[v]	T-type	20	2594-2613	GGAATGACTTTGGTCTAGTC	
P2612[c]	T-type	28	2593-2612	tcggatccACTAGACCAAAGTCATTCCG	*Bam* HI
P2639[c]	T-D1	20	2620-2639	ATTGTCTCCAATTCA TTTTG	
P2640[v]	T-D1	19	2640-2658	CAAACTTGCTAAATGAATT	
P2713[v]	T-D1	27	2713-2737	ctGAATTCTCAAAGGTAAATAATTCAA	*Eco* RI
P2713[v]	T-type	27	2713-2737	ctGAATTCTCAAAAGTAAATAATTCAA	*Eco* RI
P2718[c]	T-D1	27	2694-2718	ctGAATTCTCCAAATTACCAAAATGCC	*Eco* RI
P2718[c]	T-type	27	2694-2718	ctGAATTCTCCGAATTACCAAAATGCC	*Eco* RI
P91[c]	P-D	27	67-91	ccGGATCCCTGAAGCGCTCCTCCGAGC	*Bam* HI
P92[c]	P-type	27	68-92	ccGGATCCCTGAAGCGCTCCTCCGAGC	*Bam* HI
P87[v]	P-type	26	87-110	tcGGATCCCCGGGGAAACCTGGAGCG	*Bam* HI
P273[c]	P-type	18	256-273	GTTTCCACCGGGTAGTAG	
P271[v]	P-D	22	271-292	AACTGAAGCTCCCGAGAACCGC	
P274[v]	P-type	22	274-295	AACTGAAGCTCCCGAGAACCGC	

*P-D: PSTVd Darwin strain; P-type: PSTVd type strain; T-type: TLCV type strain; T-D1: TLCV Darwin1 strain

[a]Nucleotide position of TLCV primers as in Appendix A, PSTVd type strain as in *Gross et al.* (1978) and PSTVd-D as in Appendix B

[b]Lowercase letters indicate extra nucleotide residues including restriction sites introduced for cloning and/or for ^{32}P-end-labelling of fragments after digestion with restriction enzymes.

[c]Complementary-sense strand primers

[v]Virion-sense strand primers

2.2 METHODS

Standard molecular biology methods used were as described in Sambrook *et al.* (1989) or from manufacturers of the kits where specified. Other general methods used in this study are described here and specific methods are outlined in relevant chapters.

2.2.1 Growth of plants

Tomato (*Lycopersicon esculentum*, var. Grosse Lisse), datura (*Datura stramonium*) and *Nicotiana benthamiana* were grown in an insect-proof glasshouse at a temperature ranging from 15 to 28°C.

2.2.2 Viral inoculation

Mechanical inoculation was carried out by rubbing sap from infected plants onto leaves of young seedlings dusted with Carborundum. Inoculation by grafting was performed by inserting a scion of an infected plant into a downward cut in the stem of a stock plant and bound together with a parafilm strip. Agroinoculation was carried out by injecting 10 μl of *A. tumefaciens* strain C58 (Section 2.2.12) cultures into the multiple sites near axillary buds of the main stem of plants.

2.2.3 DNA extraction

Plant tissues (0.1 g or 10 g) were pulverised in liquid nitrogen, mixed with two volumes of DNA extraction buffer containing 50 mM Tris-HCl pH 8, 100 mM NaCl, 10 mM EDTA, 1% (w/v) SDS and 1% (v/v) β-mercaptoethanol. The mixture was extracted four times with an equal volume of phenol:chloroform (4:1) and once with an equal volume of chloroform:isoamylalcohol (24:1). The nucleic acids were precipitated by addition of $^{1}/_{10}$th volume of 3 M sodium acetate, pH 5, and 2.5 volumes of ice-cold

ethanol, followed by incubation at -20°C for 1 h. The precipitate was recovered by centrifugation in a micro-centrifuge at 11,300 *g* for 10 min or in Swing-bucket rotor (eppendorf, Germany) at 3,969 *g* for 15 min at room temperature. Pellets were washed in 80% ethanol prior to drying in a vacuum and resuspended in TE buffer (Table 2.1). The suspension was incubated with 10 μg μl^{-1} ribonuclease A at 37°C for 1 h. The ribonuclease was removed by phenol:chloroform extraction. The DNA was reprecipitated with ethanol and resuspended in water at a rate of 250-400 μl for each g of tissue extracted. DNA samples (1-5 μg) were either analysed by southern blot hybridisation (2.2.17) or diluted 10 fold in water and 1 μl containing approximately 150 ng DNA used in polymerase chain reaction (2.2.7).

2.2.4 Restriction enzyme digestion of DNA

DNA was digested with restriction endonucleases from Boehringer Mannheim (Germany) and Promega (USA) using buffer systems recommended or supplied by the manufacturers.

2.2.5 Gel electrophoresis

2.2.5.1 Agarose gel electrophoresis

Agarose gels were prepared from 0.7-2.0% (w/v) solutions of Type I-A: Low EEO agarose containing 0.5 μg/ml (w/v) ethidium bromide in TBE buffer. Horizontal mini-gel electrophoresis systems (OWL Scientific, Inc., Cambridge, models B2 or B1A) were used. DNA samples were adjusted to 1x loading buffer before applying to the wells. DNA molecular weight markers III and IV (Boehringer Mannheim) were used as high range molecular weight markers and DNA molecular weight marker VI as a low range molecular weight marker. Gels were electrophoresed in TBE running buffer and

photographed using a short wavelength UV transilluminator.

2.2.5.2 Polyacrylamide gel electrophoresis

Denaturing polyacrylamide gels for sequencing and DNA footprinting contained 6 or 8% (w/v) acrylamide, 0.32% (w/v) bisacrylamide, 8 M urea, 0.09 M Tris-HCl, 0.09 M Borate and 2 mM EDTA. Polymerisation was initiated by the addition of 1.25 mg ml^{-1} freshly prepared ammonium persulfate and 50 µl of TEMED per 50 ml of solution. The solution was poured between glass plates of 20 x 40 x 0.05 (or 0.02) cm. Gels were allowed to set for at least 30 min, then pre-electrophoresed at high voltage (1200-1500 V) until gel temperature was approximately 50°C. DNA samples were electrophoresed at 50 W (45-50°C) after denaturing of samples in formamide loading buffer by heating at 80°C for 2 min.

Non-denaturing polyacrylamide gels (20 x 20 x 0.05 cm) for electrophoretic mobility shift assays were prepared from 40 ml solutions containing 4% (w/v) acrylamide, 0.16% (w/v) bisacrylamide in TBE buffer. DNA samples were electrophoresed at 20 mA for 2.5 h.

2.2.5.3 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE)

SDS PAGE was conducted as described essentially by Laemmli (1970) using a mini-gel system (Hoeffer Mighty Small II, San Francisco, USA). The gel was comprised of a 4% stacking layer (buffered in 125 mM Tris-HCl, pH 6.8 and 0.1% SDS) and a 10% resolving layer (buffered in 380 mM Tris-HCl, pH 8.8 and 0.1% SDS) in a discontinuous buffer system (Table 2.1). Protein samples were heated at 100°C for 3 min in a SDS-gel loading buffer (Table 2.1) and electrophoresed in SDS-PAGE running buffer at 100 V until the bromophenol blue had reached the bottom of the gel.

Gels were stained for 2 h in 0.125% Coomassie brilliant blue R-250 in 50% methanol and 10% glacial acetic acid and destained first in 50% methanol and 10% acetic acid for 2 h and then overnight in 7% methanol and 5% acetic acid.

2.2.6 Purification of DNA from gel slices

2.2.6.1 From agarose gels

DNA bands excised from agarose gels after staining with ethidium bromide were extracted from the gel by the Geneclean procedure, using kits supplied by Bio101 (USA) or Bresatec (Australia), or alternatively with a QIAquick gel extraction kit (QIAGEN, Germany) according to the manufacturer's instructions.

2.2.6.2 From polyacrylamide gels

The volume of gel slices containing DNA was estimated, 2 volumes of elution buffer (Table 2.1) added and incubated at 37°C overnight. The solution was transferred to a fresh microfuge tube, 2 volumes of ice-cold ethanol added and stored at -20°C for 1 h. The DNA was recovered by centrifugation at 52,000 *g* for 30 min in a Beckman TL-100 Ultracentrifuge and resuspended in water.

2.2.7 DNA amplification

The polymerase chain reaction (PCR) was carried out in a volume of either 20 or 50 μl containing 1x reaction buffer (50 mM KCl, 10 mM Tris-HCl pH 9.0 at 25°C, 0.1% Triton X-100), DNA template, oligonucleotide primers (each at 1 μM), 200 μM each of dCTP, dGTP, dTTP and dATP, 1.5 mM $MgCl_2$ and 50 U ml^{-1} of Taq DNA polymerase (Promega). The mixture was subjected to a 30 cycle PCR program consisting of 1 min at 94°C, 2 min at 55°C, and 3 min at 72°C. The final cycle was

followed by a 7 min incubation at 72°C.

Where necessary, the PCR products were purified with a QIAquick PCR purification kit (QIAGEN, Germany) according to the manufacturer's instructions.

2.2.8 Dephosphorylation of vector DNA

To prevent self-ligation of linearised vectors, the 5′-phosphate group was removed using calf intestinal alkaline phosphatase (CIP, Boehringer Mannheim) in a reaction containing restricted DNA vector and the reaction buffer provided by the same source. The reaction was carried out at 37°C for 1 h, then an equal volume of 10 mM EDTA was added and DNA was purified by phenol:chloroform extraction and ethanol precipitation.

2.2.9 End-filling of DNA fragments

End-filling of dsDNA fragments with 3'-recessed ends for blunt-end cloning was performed with T4 DNA polymerase (Boehringer Mannheim) in a reaction containing 250 μM each dNTP and the reaction buffer provided by the same source. The reaction was incubated at 37°C for 30 min, then terminated by incubation at 65°C for 10 min. DNAs were purified by phenol:chloroform extraction and ethanol precipitation.

2.2.10 DNA ligation

Linearized vector (20-50 ng) was ligated with purified DNA fragment in molar ratios of approximately 3:1 (insert:vector respectively). The ligation was carried out in a volume of 10 μl containing DNA insert, vector DNA, 2 mM ATP, 1 U T4 DNA ligase (Boehringer Mannheim) and 1x ligation buffer provided by the same source. The mixture was incubated at 16°C overnight and used directly (1 μl) to electroporate

bacterial cells (see below). When a larger amount of DNA was required for electroporation the ligation reaction was concentrated by N-butanol extraction. N-butanol precipitation was carried out as outlined in Table 2.3 and 5 μl of the DNA was used for electroporation.

***Table 2.3** N-butanol precipitation*

1. Denature T4 DNA ligase by incubation of the ligation mixture at 65°C for 10 min.
2. Dilute the reaction to 60 μl with water.
3. Add 500 μl N-butanol and vortex for 5 sec.
4. Centrifuge at 11,300 *g* for 10 min at room temperature.
5. Discard the N-butanol.
6. Resuspend the pellet in 10 μl water.

For rapid ligation, desired DNA fragments were excised from 1.0-1.5% NuSieve low melting point agarose (FMC Bioproducts, USA) gels. The gel slices were melted at 68°C for 10 min and placed at 37°C. The molten agarose (7 μl) was mixed with 3 μl of water (at 37°C), 1.5 μl 10x ligase buffer, 1 μl T4 DNA (Boehringer Mannheim), 1.5 μl ATP (5 mM) and 1 μl vector and left at 37°C for 30 seconds, before incubation at room temperature for 2 hours. The ligation mixture was then reheated to 68°C, placed at 37°C and 1 μl used for transformation by electroporation.

2.2.11 Transformation of bacteria with recombinant plasmids

Transformation of bacteria with plasmids was performed by electroporation, using a Gene-Pulser apparatus (Bio-Rad, USA). The procedure used to prepare electrocompetent cells was essentially described in the Bio-Rad manual. The protocol is outlined in Table 2.4.

Table 2.4 *Preparation of electrocompetent cells.*

1.	Inoculate 500 ml of LB-broth with 5 ml of an overnight bacterial culture.
2.	Grow cells in a 2 litre flask either at 37°C (*E. coli*) or at 28°C (*A. tumefaciens*) with vigorous shaking to an optical density (A_{600}) of 0.5.
3.	Chill the flask on ice for 10 min.
4.	Centrifuge in 250 ml buckets at 4,000 *g* for 15 min at 4°C.
5.	Discard the supernatant and resuspend the cells gently in 500 ml of ice-cold water.
6.	Centrifuge as step 4.
7.	Resuspend in 250 ml of ice-cold water.
8.	Centrifuge as step 4.
9.	Resuspend in 10 ml of ice-cold water and transferr to a sterile 50 ml tube
10.	Centrifuge as above. Carefully remove supernatant.
11.	Resuspend pellet in ice-cold 10 % glycerol to a final volume of 2 ml.
12.	Aliquot 40 µl into an ice-cold Eppendorf, snap freeze in liquid nitrogen, and store at -80°C.

Plasmid DNA for electroporation (see 2.2.10 and 2.2.13) was mixed with 40 µl electrocompetent cells and transferred to an ice-cold cuvette (Bio-Rad) with 0.1 cm electrode gap. The cuvette was placed into the Gene-Pulser electroporator set at 1.8 kV, 25 µFD and 200 Ohm and given a single pulse immediately. The cells were resuspended in 1 ml LB media and incubated either at 37°C for 1 h (*E. coli*) or at 28°C for 3 h (*A. tumefaciens*). Aliquots of 50-200 µl cell culture were spread onto agar plates containing suitable antibiotic and incubated either overnight at 37°C (*E. coli*) or 48-72 h at 28°C (*A. tumefaciens*). Blue-white colony selection of *E. coli* cells was achieved by adding 25 µg ml^{-1} IPTG and 40 µg ml^{-1} X-Gal to the agar medium.

2.2.12 Growth of bacteria

Liquid cultures were set up by inoculating LB broth with single bacterial colony. Cultures of *E. coli* were incubated at 37°C overnight and cultures of *A. tumefaciens* were incubated at 28°C for 48-72 h with shaking. As appropriate, the growth media

contained the antibiotics ampicillin (100 μg ml^{-1}), kanamycin (25 or 50 μg ml^{-1}) and rifampicin (50 μg ml^{-1}).

2.2.13 Preparation of plasmid DNA

Minipreparation of plasmid DNA was routinely performed using the boiling method according to Holmes and Quigley (1981) with some modifications.

Bacteria were pelleted from 2 ml of overnight culture by centrifugation in a micro-centrifuge at 11,300 *g* for 1 min at room temperature. The supernatant was discarded and the pellet resuspended in 0.35 ml of STET buffer (Table 2.1). After adding 12.5 μl of a 20 mg ml^{-1} lysozyme stock the suspension was placed in a boiling-water bath for 1 min and then centrifuged for 30 min at room temperature as described above. The gelatinous pellet was removed with a toothpick and nucleic acid precipitated from the supernatant by the addition of 220 μl ice-cold isopropanol and 40 μl 3.0 M sodium acetate, pH 5.0. The DNA was recovered by centrifugation, washed with 500 μl ice-cold 80% ethanol and dried under vacuum for 10 min. The pellet was resuspended in 30 μl of either water or TE buffer.

For sequencing, plasmid DNA obtained from a 5 ml culture (as outlined above) was extracted twice with phenol:chloroform (4:1) and once with chloroform:isoamylalcohol (24:1). The DNA was precipitated with ethanol and resuspended in 25 μl water. The concentration of DNA was estimated by spectrophotometry.

Large scale (150-300μg) preparation of plasmid DNA was carried out using the QIAGEN Maxi Kit according to the manufacturer's instructions.

2.2.14 DNA sequencing

The dideoxynucleotide chain termination sequencing method (Sanger *et al.,* 1977) was used on ds plasmid DNAs, either manually using a Sequenase version 2.0 DNA sequencing kit (United States Biochemical) according to the manufacturer's instructions or using an ABI PRISM system at the Department of Haematology, Flinders Medical Centre, Adelaide, Australia. For manual sequencing, dsDNA templates were prepared as described (Section 2.2.13) and denatured by the addition of 2 µl of 2 M NaOH to 18 µl of plasmid DNA (about 16 µg DNA). Denatured DNAs were reneutralized by passage through micro-spin columns of 0.5 ml bed volume containing Sephadex G-50 resin (Pharmacia). Approximately 4 µg of denatured DNA was used in each sequencing reaction.

2.2.15 Preparation of ^{32}P-labelled probes

2.2.15.1 Labelling of DNA

^{32}P-labelled probes were synthesised by random priming using a GIGAprime DNA labelling kit (Bresatec, South Australia). A full-length TLCV DNA-template (20 ng) was denatured by heating at 95°C for 5 mins and cooled on ice. Reactions of 24 µl volume contained denatured DNA, 50 µCi α-^{32}P-dATP (3000 Ci/mmol), 500 ng random decamer oligodeoxyribonucleotides, 5 U of klenow fragment of *E. coli* DNA polymerase I (Bresatec, Australia), 160 µM of each of the non-radioactive nucleotides dCTP, dTTP and dGTP in a buffer containing 200 µg ml^{-1} BSA, 100 mM Tris-HCl pH 7.6, 20 mM $MgCl_2$, and 20 mM NaCl. Labelling reactions were incubated at 37°C for 30 min and terminated by the addition of EDTA pH 8.0 to a final concentration of 20 mM.

Unincorporated dNTPs were removed by passing the probe through a 1 ml syringe containing Sephadex G 50 resin previously equilibrated with 1 ml of TE buffer. Labelled DNA probe was collected from the column in the void volume.

2.2.15.2 End-labelling using Klenow fragment

PCR products or restriction fragments (100-150 ng) with 3' recessed ends were end-labelled using klenow fragment in the same reaction outlined in section 2.2.15.1 except that the decanucleotide solution was omitted from the reaction. After the removal of unincorporated dNTPs (Section 2.2.15.1), 1 μl of the end-labelled probe used in electrophoretic mobility shift assay reactions (see section 4.2.4)

2.2.15.3 End-labelling of oligodeoxyribonucleotides using polynucleotide kinase

Primers were 5′-end-labelled in a reaction volume of 5.0 μl containing 100 ng primer, 25 μCi γ-^{32}P-ATP (4000 Ci/mmol), 5 mM DTT and 5.0 U of T4 polynucleotide kinase (Promega) in the reaction buffer supplied by the same source.

2.2.16 Southern blot hybridisation analysis

DNA samples were subjected to electrophoresis in 1.2% agarose gels in TBE buffer containing 0.5 μg ml^{-1} ethidium bromide and blotted onto a Zeta-Probe nylon membrane (Bio-Rad) by a rapid downward transfer system (Schleicher & Schuell, New Hampshire) according to the manufacturer's instructions. The DNA was cross-linked to the membrane using a Stratalinker UV source (Stratagene, La Jolla, CA) and hybridised overnight with a ^{32}P-labelled TLCV probe prepared by random priming (2.2.15). The membrane was washed twice with 2x SSC, 0.1% SDS, each time for 5 min at room temperature and twice with 0.1x SSC, 0.1% SDS, each time for 15 min at 65°C. The

positions of radioactive bands were determined by autoradiography.

2.2.17 Dot blot analysis

Dot blot analysis was carried out as described by Maule *et al.* (1983). Fifty mg of leaf tissue was ground in liquid nitrogen and extracted in 100 µl TE buffer. Tissue homogenate was diluted 1:1 with 1 M NaOH and incubated at room temperature for 30 min. After centrifugation at 11,300 g for 10 min, 5 µl of supernatant was spotted onto a Zeta-Probe nylon membrane. The membrane was soaked briefly (20 sec) in 2x SSC, exposed to UV light and subjected to hybridisation as outlined in 2.2.16.

Chapter Three

New Potato Spindle Tuber Viroid and Tomato Leaf Curl Geminivirus Strains from a Wild *Solanum* sp.

3.1 INTRODUCTION

In Australia, field infections of TLCV are currently limited to early or mid-season tomato crops in the Northern Territory where indigenous populations of the whitefly *B. tabaci* occur. The disease has appeared each year since 1970 causing complete crop failure in some very early plantings in coastal areas (Condé and Connelly, unpublished data). The continued severity of early season infections, together with bacterial wilt, has made tomatoes unprofitable in the Darwin area. Infected tomato plants show varying degrees of stunting depending on how early they were infected, as well as leaf curling, yellowing, upward leaf rolling and cessation of fruit production. Tobacco (*Nicotiana tabacum*), eggplant (*Solanum melongena*), datura (*Datura stramonium*) and zinnia (*Zinnia elegans*) are also natural hosts of the virus (Conde and Connelly, 1994).

In an effort to identify the natural reservoir of TLCV in the Northern Territory, various solanaceous and non-solanaceous wild plants were tested for the presence of TLCV. Tomato plants graft-inoculated with scions collected from plants of a wild *Solanum* sp. from Darwin were found to contain TLCV-like viruses but displayed marked symptom differences compared to those caused by the type strain of TLCV (Condé and Connelly, 1994; Dry *et al.*, 1993). In this chapter, the isolation of two new strains of TLCV in a mixed infection with a new strain of potato spindle tuber viroid (PSTVd) is reported and their molecular relationships with the respective type strains of TLCV and PSTVd are analysed.

3.2 MATERIALS AND METHODS

3.2.1 Disease source and transmission tests

Scions were collected from several plants of the wild *Solanum* weed and grafted individually to cherry tomato (*L. esculentum* var. *cerasiforme*) plants in Darwin. Five of these tomato plants developed delayed symptoms consisting of elongated shoots and a feathery appearance. Scions from two of these symptomatic cherry tomatoes were grafted onto two tomato plants (var. Grosse Lisse) in Adelaide and served as disease sources. These infected tomato plants, referred to as Darwin 1 (D1) and Darwin 2 (D2), were used as inoculum sources to inoculate other plants by grafting or by mechanical inoculation as described in section 2.2.2. TLCV infected plant tissues following dodder (*Cuscuta campestris*) inoculation were those described by Behjatnia *et al*. (1996).

3.2.2 DNA extraction and analysis

The viral DNAs were extracted as described (Section 2.2.3) and analysed by either southern blot (Section 2.2.16) or dot blot (Section 2.2.17) hybridisations.

3.2.3 Cloning and sequencing of geminivirus DNAs

A pair of TLCV type strain oligonucleotide primers, P169^{c} and P2339^{v} (Table 2.2), was used to prime the amplification of DNA fragments of the new TLCV strains. These fragments of approximately 560 bp were blunt-end ligated into the *Eco* RV site of the plasmid Bluescript II SK^{+} and sequenced using the dideoxynucleotide chain termination method (Section 2.2.14). Specific primers to new strains were designed from the sequence of the 560 bp fragment and used to amplify and clone the full-length DNA of new strains (see section 3.3.4). Sequence alignment was carried out using the program NALIGN from the PC/GENE sequence analysis software package

(IntelliGenetics Inc., Mountain View, CA).

3.2.4 Nuclease digestions

Total leaf nucleic acids were extracted from mechanically-infected tomato plants as described in section 2.2.3 up to and including ethanol precipitation. The nucleic acids were then treated with either 10 μg μl^{-1} of DNase-free RNase A (Boehringer Mannheim) in TE buffer and/or with 10 μg μl^{-1} of RNase-free DNase (type DN-EP, Sigma, St. Louis, Missouri) in 10 mM Tris-HCl, pH 7.5, containing 10 mM $MgCl_2$. The samples were incubated at 37°C for 1 h and used for mechanical inoculation.

3.2.5 Viroid extraction, purification and analysis

Viroid RNA was extracted from infected material and purified as described by Rezaian *et al.* (1988). Nucleic acids extracted from 50 g tissue were purified by chromatography on cellulose CF-11 and enriched for soluble RNA species by salt fractionation (Rezaian *et al.,* 1988). RNAs present in the 2 M LiCl soluble fraction were concentrated by ethanol precipitation and the viroid species was separated from other nucleic acids by 2-dimensional polyacrylamide gel electrophoresis (Schumacher *et al.,* 1983). The circular viroid RNA band was excised and eluted from the gel (Rezaian *et al.,* 1988), recovered by ethanol precipitation and suspended in 30 μl TE buffer.

Samples of viroid RNA were subjected to dot blot hybridisation as described by Rezaian *et al.* (1988), using a PSTVd specific probe synthesised from a full-length PSTVd cDNA clone (supplied by Professor Bob Symons, Adelaide University, South Australia) as described in section 2.2.15.1. Purified circular viroid RNA (3μl) was mixed with 3 vol of 10x SSC containing 20% formaldehyde. The mixture was heated at 65°C for 15 min, then at 90°C for 2 min and applied to the Zeta-Probe membrane. The

RNA was cross-linked to the membrane by exposing to UV light for 1 min. Hybridisation, washing and autoradiography were carried out as described in section 2.2.16.

3.2.6 Viroid cDNA synthesis, cloning and sequencing

Two different sets of oligonucleotide primers were used for reverse transcription and amplification of the viroid template. The first set, $P273^c$ and $P274^v$ (Table 2.2), was selected from published PSTVd sequence (Gross *et al.*, 1978). The second set of overlapping primers was designed to a conserved *Bam* HI site which is also present in PSTVd and had two non-PSTVd nucleotide residues at their 5′-ends. These primers, $P92^c$ and $P87^v$ (Table 2.2), were used to confirm the sequences of regions selected for the first set of primers.

Purified circular RNA (3µl) was mixed with 2 µl of either $P273^c$ or $P92^c$ at a concentration of 10 µM, heated to 95°C for 3 min and quickly cooled on ice. Reverse transcription of viroid RNA was carried out in a 15 µl reaction containing the template and primer, 50 mM Tris-HCl (pH 8.3 at room temperature), 10 mM $MgCl_2$, 25 mM KCl, 500 µM each of dCTP, dGTP, dTTP and dATP, 1 mM DTT, and 4 units of AMV reverse transcriptase (Promega). The mixture was incubated at 42°C for 15 min. PCR was carried out in a 50 µl reaction containing 5 µl of cDNA reaction, $P273^c$ and $P274^v$ or $P92^c$ and $P87^v$ (each at 1 µM) using the conditions described in section 2.2.7.

PCR fragments were cloned into a T-tailed Bluescript II SK^+ plasmid prepared as described (Section 2.1.3). Two independent full-length PSTVd clones were sequenced in both directions using the dideoxynucleotide chain termination method (Section 2.2.14). Multiple alignment of viroid sequences was carried out using the program

PILEUP (Genetics Computer Group, University of Wisconsin).

3.3 RESULTS

3.3.1 Detection of TLCV like-DNAs

Symptoms of the two infected source tomato plants, D1 and D2, differed from each other. D1 displayed leaf curling, yellowing and upward leaf rolling suggesting it contained a TLCV-like geminivirus. However, D2 showed bunched growth with distorted leaves in initial growth, followed by spindly and hairless shoots and leaf narrowing at later stage. Dot blot analysis using a TLCV type strain probe gave a positive signal with the D1 tomato but not with the D2 tomato plant (data not shown). A more sensitive analysis for the presence of TLCV-like species was carried out using PCR with two TLCV-specific primers designed to direct the amplification of a fragment composed of the TLCV intergenic region and the 5′ end of the C1 ORF (Dry *et al.*, 1993). PCR fragments corresponding to the expected 562 bp DNA product of tomato plant infected with the TLCV type strain were obtained with extracts from both D1 and D2 plants (Fig. 3.1). However, the level of amplified DNA from the D2 plant extract was significantly lower than that obtained with extracts from D1 and the wild type TLCV infected plants. This observation was consistent with the lack of a positive signal with the D2 plant extract in the dot blot assay. These experiments showed that the D1 and D2 tomato plants contained TLCV-like DNA.

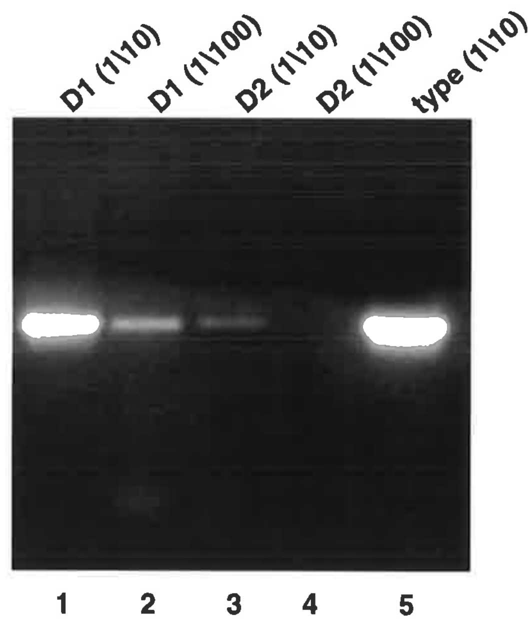


Fig. 3.1 *Amplification of TLCV DNA from total nucleic acid extracts of D1, D2 and type-infected tomato plants.*

Lanes 1 and 2, D1 extract diluted 10 and 100 times, respectively; lanes 3 and 4, D2 extract diluted 10 and 100 times, respectively; lane 5 TLCV type strain extract diluted 10 times.

3.3.2 Isolation of the mechanically transmissible agent

To resolve the symptom differences between D1 and D2 tomato plants, mechanical disease transmission from both plants was examined. Tomato plants mechanically inoculated with sap from both sources produced disease symptoms which were similar and consisted of a zone of stunted growth with shorter, distorted leaves which emerged 7-10 days after inoculation (Fig. 3.2A). The next phase of symptom development occurred 2-3 months after inoculation and produced a zone of elongated spindly shoots, smaller leaves and hairless stems (Fig. 3.2 B & C). Necrotic streaking of the stem, vein necrosis of leaflets and small seedless fruit (Fig. 3.2D) were also characteristic symptoms. Dot blot and PCR analyses using a TLCV-specific probe and primers, respectively, did not detect any TLCV-like DNA species in these plants indicating that these symptoms were not due to TLCV.

Seventeen datura plants mechanically inoculated with sap of D1 and D2 tomato plants did not produce symptoms. However, inoculation of extracts of these symptomless datura plants back onto healthy tomato plants produced typical symptoms of the mechanically transmissible agent showing that datura is a symptomless host of this agent. *Nicotiana tabacum, Solanum melongena, S. torvum, S. mauritianum,* and *Capsicum annum* were also found to be symptomless hosts of the mechanically transmissible agent. These experiments indicated that both D1 and D2 tomato plants contained a TLCV-like virus together with a mechanically transmissible agent.

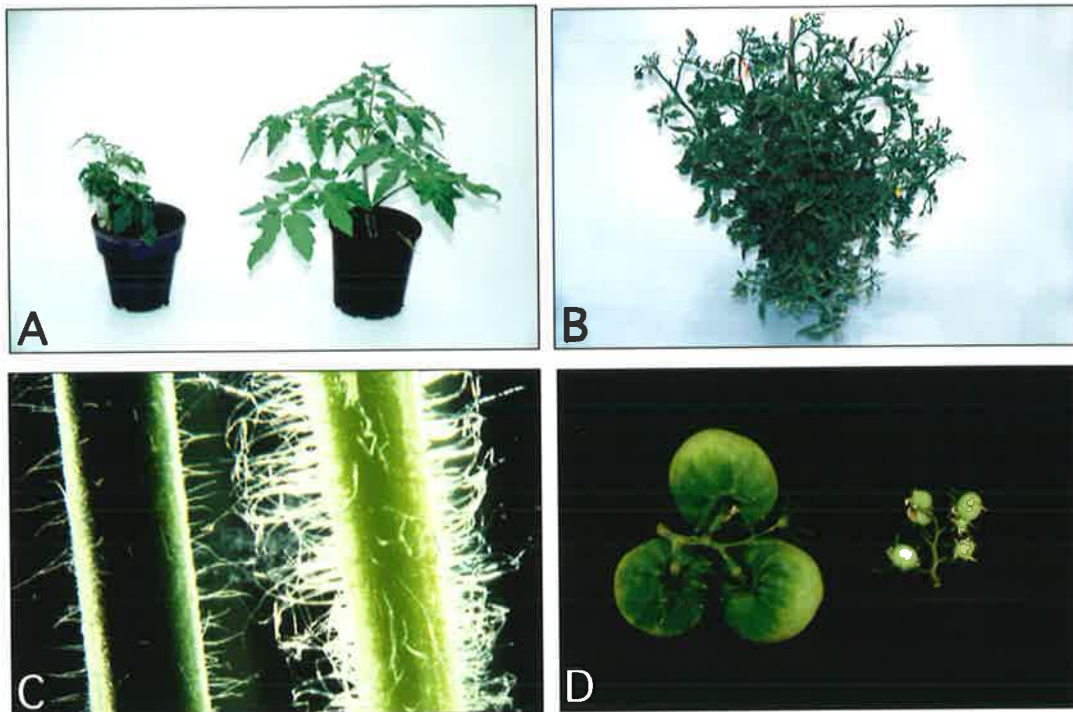


Fig. 3.2 *Symptoms caused by the mechanically transmissible agent in tomato plants.*

A) Strong epinasty of leaves and leaflets and bunched growth 7-10 days after inoculation (left) compared with healthy plant (right). B) Spindly shoots and small leaves 2-3 months after inoculation. C) Hairless stem of infected plant (left) compared with healthy stem (right). D) Small fruit produced by infected plant(right)compared with normal fruit of healthy plant (left).

3.3.3 Separating the TLCV-like viruses from the mechanically transmissible agent

Dodder transmission was used to resolve the disease complex. A group of 10 tomato plants were inoculated with D2 tomato plant by dodder. Two of these plants developed symptoms characteristic of TLCV, another two plants displayed symptoms characteristic of the mechanically transmissible agent and the other six plants remained symptomless. Both receptor plants showing TLCV-like symptoms lacked the mechanically transmissible agent as the extracts of these plants failed to produce any symptoms when mechanically inoculated onto 11 healthy tomato plants and observed for 30 days. However, all 8 healthy tomato plants mechanically inoculated with the sap of 2 receptor plants with symptoms associated with the mechanically transmissible agent showed characteristic symptoms of this agent after 30 days. This experiment showed that dodder separated TLCV-like virus from the mechanically transmissible agent, which co-existed in the D2 plant. Although the titre of the geminivirus in the original D2 tomato plant was only detectable by PCR (Fig. 3.1), the virus could be readily detected by dot blot hybridisation in tomato plants infected via dodder transmission. This observation raised the question as to whether there may be an interaction between the TLCV-like viruses and the mechanically transmissible agent. To test this possibility tomato and datura plants were first inoculated with the mechanically transmissible agent and then with scions of tomato plant infected with TLCV-like virus transmitted from the D2 tomato plant by dodder. Both agents were detectable in inoculated tomato plants after 30 days indicating that there was no inhibitory interaction between them during that period. The low level of geminivirus DNA in the original D2 plant may relate to the age of infection and/or the presence of an unidentified viral component.

3.3.4 Sequence analysis of TLCV D1 and D2 strains

A pair of TLCV D1-specific oligonucleotide primers, P2639^c and P2640^v (Table 2.2), designed from the partial sequence of TLCV D1 was used to amplify a full-length DNA of the TLCV D1 and D2. Full-length PCR products of the TLCV strains were subjected to restriction enzyme digestions (Sambrook *et al.,* 1989). A unique *Kpn* I site was identified within the DNA sequence of these strains. These DNA products were blunt-end ligated into the *Eco* RV site of the plasmid Bluescript II SK$^+$, and a region of each DNA containing the *Kpn* I site was sequenced. Two TLCV D1 oligonucleotide primers containing an overlapping *Kpn* I site, P1588^c and P1583^v (Table 2.2), were used to re-clone full length monomeric D1 and D2 DNAs into a Bluescript II SK$^+$. The D1 clone was infectious (see Chapter 5). Both strands of these clones were sequenced using 10 different TLCV oligonucleotides primers. Sequence analysis of the TLCV D1 (2766 nt) and D2 (2767 nt) showed them to have the same genome organisations as the 2766 nt TLCV type strain (Fig. 1.4 and Dry *et al*., 1993).

The nucleotide and amino acid sequence comparisons between the TLCV strains are shown in Table 3.1 (see also Appendix A). D1 and D2 have an overall nucleotide sequence similarity with the TLCV type strain of 94.8 and 94.1%, respectively, and show 96.2% overall sequence similarity to each other. Nucleotide sequence similarity between corresponding ORFs of the TLCV type strain and either D1 or D2 is in the range 92.9-99.4%. The C1 ORF encoding the viral Rep are least similar (92.9%). D1 and D2 show 85.9 % sequence similarity with the TLCV type strain in the intergenic region (IR), a highly variable region in whitefly transmitted geminivirus genomes (Padidam *et al.,* 1995a) and only 79.5-80.1% in the left part of this region (Table 3.1).

The translation products of the virion-sense ORFs V1 and V2 are almost identical to those of the type strain, with only 3 and 1 amino acid differences respectively. However, differences between the products of the complementary-sense are more significant (Table 3.1). Twenty amino acid differences exist in the translation product of the C1 ORF of new and type strains and these differences mostly occur at the N-terminus (Fig. 3.3).

Table 3.1 Sequence comparison between TLCV strains

		% identity					
	nucleotides	Type vs D1		Type vs D2		D1 vs D2	
ORFs	(start - stop)	nt seq.	aa seq.	nt seq.	aa seq.	nt seq.	aa seq.
C1	2615 - 1530	93.9	94.5	92.9	94.5	97.2	98.1
C2	1627 - 1223	97.1	94.1	96.6	94.8	96.3	93.3
C3	1479 - 1078	96.8	94.0	96.5	94.0	96.3	94.0
C4	2464 - 2159	97.1	96.1	98.1	98.0	99.0	98.0
V1	148 - 492	98.9	98.3	99.4	99.1	98.6	97.4
V2	308 - 1075	97.5	99.6	96.4	99.2	94.7	98.8
DNA region							
Full-length	1 - 2766	94.8		94.1		96.2	
IR	2616 - 147	85.9		85.9		95.3	
Left part of the IR	2616 - 2766	80.1		79.5		99.3	

```
Strains

TYPE  - MTRPKSFRINAKNYFLTYPKCSLTKEEALSQLNNLETPTSKKYIKVCREL -50
D1    - .AP..R...Q...F.........S........QS.N...N...L.I.... -50

TYPE  - HENGEPHLHVLIQFEGKFQCKNQRFFDLVSPTRSAHFHPNIQGAKSSSDV -100
D1    - .................YV.T..................T.....G.... -100

TYPE  - KSYLEKDGDTLEWGEFQIDGRSARGGQQSANDAYAQALNTGSKSEALNVL -150
D1    - .................................................. -150

TYPE  - RELAPKDYVLQFHNLNSNLDRIFTPPLEVYVSPFLSSSFDRVPEELEEWV -200
D1    - ........................L........S..........V..... -200

TYPE  - AENVKDAAARPLRPISIVIEGESRTGKTVWARSLGPHNYLCGHLDLSPKV -250
D1    - .................................................. -250

TYPE  - YSNDAWYNVIDDVDPHYLKHFKEFMGAQRDWQSNTKYGKPVQIKGGIPTI -300
D1    - .................................................. -300

TYPE  - FLCNPGPNSSYKEYLDEEKNSALKAWALKNAEFITLNEPLYSGTYQGPTQ -350
D1    - .................................................. -350

TYPE  - NSEEEVHPEEEN -362
D1    - ............ -362
```

Fig. 3.3 *Alignment of amino acid residues of the C1 ORFs of the TLCV type and D1 strains.*
Predicted amino acid sequences are depicted using the one-letter code designation. Dots indicate that the residues are identical with the amino acids in the top sequence.

3.3.5 The mechanically infectious agent is a viroid.

Several attempts were made to isolate virus-specific DNA or dsRNA from the mechanically transmissible viral agent but these were unsuccessful. Total nucleic acid extracts from infected plants were found to be fully infectious when mechanically inoculated onto healthy tomato plants. The infectious nucleic acid extract was treated with RNase and/or DNase and then used for mechanical inoculation of healthy plants. All 5 tomato plants inoculated with DNase-treated nucleic acid showed typical disease symptoms. However, none of the five tomato plants inoculated with nucleic acids treated with RNase or RNase + DNase displayed any symptoms. This observation

indicated that the mechanically infectious agent had an RNA genome.

Analysis of purified nucleic acid by 2-dimensional gel electrophoresis and silver staining revealed the presence of a circular nucleic acid species migrating more slowly than the plant linear RNAs, typical of a viroid (Schumacher *et al.,* 1983). This RNA, which was absent in healthy tomato plants (Fig. 3.4), was isolated from the gel, mechanically inoculated onto tomato plants and found to produce the same symptoms as seen in Fig. 3.2. These symptoms resembled those of PSTVd in tomato described by Diener and Raymer (1971). Dot blot hybridisation of the gel-purified RNA with a PSTVd probe under high stringency conditions produced a positive reaction suggesting that the mechanically transmissible agent was a PSTVd-like viroid. Sizing of the viroid by electrophoresis in a polyacrylamide gel under denaturing conditions showed it to be slightly smaller than citrus exocortis viroid (371 nt, results not shown).

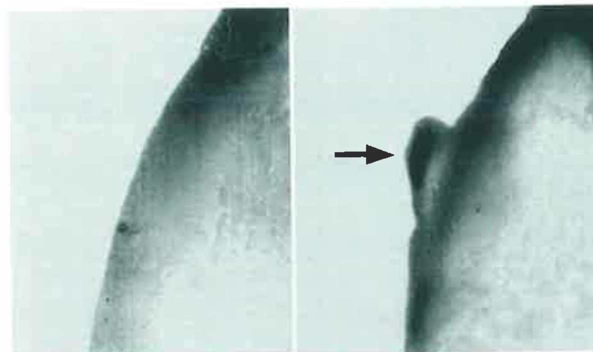


Fig. 3.4 *Analysis of nucleic acids extracts from mechanically-infected tomato plants by two-dimensional gel electrophoresis*

Purified nucleic acids from mechanically inoculated plants (right) and from healthy plants (left) were analysed and stained with silver as described in the Methods. Direction of non-denaturing electrophoresis in the first dimension was from right to left and in the second denaturing dimension was from top to bottom. The circular RNA band in infected tissue is shown by an arrow.

Primers designed from the published PSTVd sequence (P273^{c} and P274^{v}) were used in RT-PCR reactions to amplify viroid sequences. A full-length PCR product was obtained and cloned. Two of the clones were partially sequenced and a second set of primers (P92^{c} and P87^{v}) was designed from the partial sequence. These primers were used to obtain a full-length PCR product in which the sequences at the priming sites were specific to the PSTVd-like viroid isolate. The PCR product was cloned and two independent full-length clones were fully sequenced. Analysis of the data confirmed that they are indeed sequence variants of PSTVd consisting of 356 nucleotides, three nucleotides shorter than the PSTVd type strain (Gross *et al.*, 1978).

These sequence variants of PSTVd from Darwin are designated as PSTVd-D1 and PSTVd-D2. The predicted rod-like secondary structure of the PSTVd-D1 is shown in Fig. 3.5. Comparison of the primary structure of the PSTVd-D1 and PSTVd-D2 clones showed three nucleotide differences. The U residue at position 28 and two A residues at positions 252 and 346 of the PSTVd-D1 were replaced by A, G and U residues, respectively, in PSTVd-D2 (see Appendix B). These changes do not result in considerable changes to the predicted secondary structure of the PSTVd-D2. Compared to the PSTVd type strain, the PSTVd-D1 has three nucleotide deletions and 18 nucleotide exchanges (Fig. 3.5) with an overall sequence similarity of 95% with the type strain (Gross *et al.*, 1978). The sequence of PSTVd-D1 differs from those of 11 published PSTVd sequences (currently in the GenBank database) and is most similar to the Naaldwijk strain of PSTVd from the Netherlands (98.5% similarity, Puchta *et al.*, 1990). PSTVd-D1 and the Naaldwijk strain form a cluster, separate from all other strains when compared by the PILEUP program.

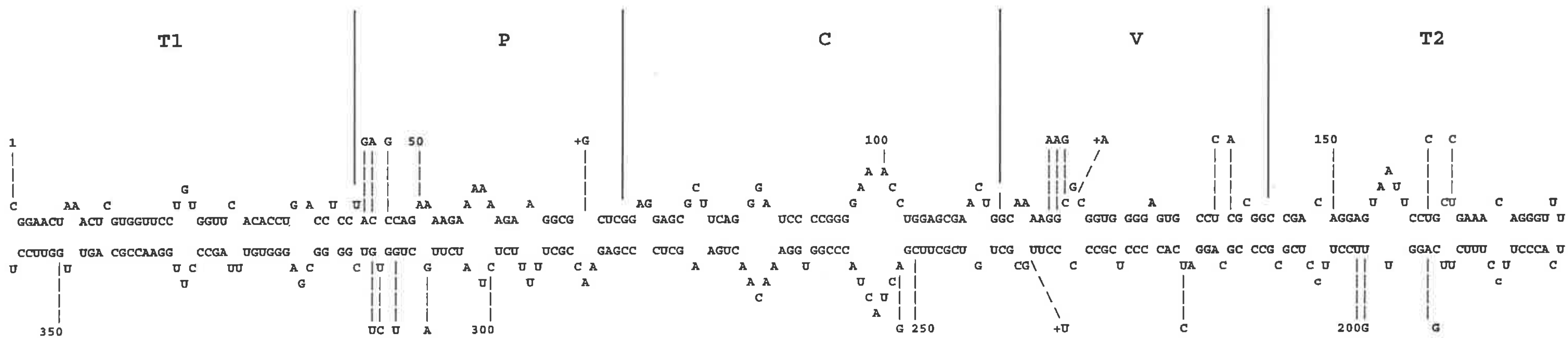


Fig. 3.5 *Nucleotide sequence and proposed secondary structure of PSTVd-D1.*

The differences in sequence as compared with the PSTVd type strain (Gross et al., 1978) are shown above. The structural domains as proposed by Keese and Symons (1985) are T1 and T2, terminal domains; P, pathogenicity domain; C, central domain; V, variable domain.

3.4 DISCUSSION

Nucleotide sequencing of two geminiviral DNAs, each derived from an individual wild *Solanum* sp. plant, showed the presence of two DNA species which were more similar to each other than to the TLCV type strain from Australia. Each new strain of TLCV showed 94-95% overall sequence similarity with the complete genome of the type strain. However, lower sequence similarities (86%) existed between the intergenic regions of the new strains and the type strain. The intergenic region is a highly variable region in whitefly transmitted geminivirus genomes, whereas the coat protein ORF is more conserved than the remainder of the genome in these geminiviruses (Padidam *et al.,* 1995a). Consistent with these observations, the greatest degree of similarity between the new and type strains was observed in the V2 (coat protein) ORF. The amino acid sequences of the complementary-sense ORFs are more variable than those of the coat protein ORF between new and type strains. The highest degree of divergence within ORFs of the TLCV strains was in the N-terminal region of the C1 ORF (Rep). Significantly, this part of the Rep protein has been shown to determine specificity of binding of Rep proteins within the intergenic region (Choi and Stenger, 1995; Jupin *et al*., 1995; Orozco *et al*., 1997).

Despite a high degree of sequence identity, geminivirus isolates often show different biological properties (Antignus and Cohen, 1994; Padidam *et al.,* 1995b). In the present study it was observed that infection of tomato plants with the TLCV type strain or the TLCV D2 strain always showed severe symptoms, whereas the TLCV D1 strain induced relatively mild symptoms.

Sequence analysis of the viroid recovered from the wild *Solanum* sp revealed that

it was a variant of PSTVd. It consists of 356 nucleotides, the same size as the Naaldwijk strain of PSTVd from the Netherlands (Puchta *et al.*, 1990) which infects tomato and pepino plants (*Solanum muricatum*). PSTVd-D, therefore, differs in size from the commonly occurring 359 nucleotides PSTVd strains. As with other strains of PSTVd (Puchta *et al.*, 1990; Schnölzer *et al.*, 1985), most nucleotide variations in PSTVd-D occur in the pathogenicity (P) and variable (V) domains (Keese and Symons, 1985), and the variations in the P domain are in the left part of this domain. Variations in the left part of the P domain have been linked to the symptom severity induced by naturally occurring sequence variants of PSTVd (Schnölzer *et al.*, 1985).

Weeds have been considered to be important reservoirs of Indian TLCV in nature (Saikia and Muniyappa, 1989). This work shows that wild *Solanum* plants may act as reservoirs for PSTVd-D and two new strains of TLCV. The recent arrival in Australia of the B biotype of *B. tabaci*, an efficient geminivirus vector (Gunning *et al.*, 1995), and its rapid spread is considered a risk to the tomato industry, particularly in Queensland, where currently about half of the Australian tomato crops are grown. The “B” biotype of *B. tabaci* has a very wide host range and may transmit the new TLCV strains from weeds including *Solanum* sp. to tomato and other host crops. It will be of interest to test the transmissibility of PSTVd-D from Solanum plants co-infected with geminiviruses, in view of the report that aphids transmit PSTVd from potato co-infected with potato leaf roll luteovirus (Salazar *et al.*, 1995).

PSTVd has not been recorded in Australia for approximately ten years and is a quarantinable disease. So far, there has been no evidence of field infection of tomato by PSTVd. There is also no evidence to indicate PSTVd-D transmission by whitefly, but

the presence of the new biotype of *B. tabaci* in the same geographical region necessitates disease monitoring for the potential spread of geminiviruses to tomato and other crops.

Chapter Four

Identification of the Replication Associated Protein Binding Domain within the Intergenic Region of Tomato Leaf Curl Geminivirus

4.1 INTRODUCTION

Geminivirus Rep-*ori* interactions are virus-specific. Lazarowitz *et al.* (1992) demonstrated that chimeric molecules derived from TGMV and SqLCV, both subgroup III geminiviruses with bipartite genomes, could replicate in *trans*, so long as the Rep and *ori* were derived from the same virus. The Logan and CHF strains of BCTV, a monopartite subgroup II geminivirus (Choi and Stenger, 1995), also possess specific replication factors which are not functionally interchangeable. Similarly, Jupin *et al.* (1995) using the Sardinian and the Israeli isolates of TYLCV, a monopartite subgroup III geminivirus, showed that Rep proteins were specific for their respective *ori*. Thus, specificity of Rep-*ori* interaction may be considered an essential trait defining individual biological species of geminiviruses (Choi and Stenger, 1996).

Sequence analysis of the intergenic region of dicot-infecting geminiviruses has identified iterative elements of 8-12 nucleotides which have been postulated to act as Rep-specific binding sites (Argüello-Astorga *et al.*, 1994). Experimentally it has been shown that the Rep-binding site of TGMV maps to a 52 bp region on the left of the intergenic region (Fontes, *et al.,* 1992). This region includes a 13-bp element (GGTAGTAAGGTAG) containing a 5-bp direct repeat which is suggested to be a high affinity binding site for the TGMV Rep (Fontes *et al.*, 1994a).

The iterative elements of the monopartite subgroup III geminiviruses, identified by sequence alignment of the intergenic region, have a different organisation compared to the Rep-binding site in TGMV. The aim of the work described in this chapter was to identify the Rep-binding domains in the monopartite DNA of TLCV. To achieve this, TLCV Rep protein was expressed in *E.* coli, purified in non-denaturing form and used

in electrophoretic mobility shift and DNase I footprinting assays. The results identified two sequence elements within a 44 nt region of the TLCV origin of replication as the Rep-binding site.

4.2 MATERIALS AND METHODS

4.2.1 Expression of TLCV Rep protein

The complete C1 ORF was amplified from a clone of TLCV (Dry *et al.*, 1993) using two TLCV-type oligonucleotide primers, P2612^{c} and P1527^{v} which contain introduced *Bam* HI and *Hin*d III sites at their respective 5′ ends (Table 2.2). The 1103 bp PCR product was digested with *Bam* HI and *Hin*d III and ligated into the expression vector pQE30 (QIAGEN, Germany). The resultant recombinant plasmid, pQE30-C1, containing the entire TLCV C1 ORF but excluding the initiation codon was fused in frame behind a vector sequence encoding an initiation methionine and six histidine residues. The identity of the clone was confirmed by restriction analysis.

Plasmid pQE30-C1 was transformed into *E. coli* strain M15 (section 2.2.11) and the cells grown overnight on an LB plate in the presence of 100 μg ml^{-1} ampicillin and 25 μg ml^{-1} kanamycin. Bacterial colonies were suspended in LB broth. The culture was grown to a cell density of 0.7-0.9 (A_{600}) at 37°C, induced with 1 mM IPTG and grown for a further 3 h at 37°C. The non-induced culture was incubated under the same conditions. The cultures were centrifuged at 10000 *g* for 2 min, the cells resuspended in SDS-gel-loading buffer (65 mM Tris HCl, pH 6.8, 2% SDS, 2.5 % β-mercaptoethanol), boiled for 3 min and electrophoresed in a 10% SDS-polyacrylamide gel as described in section 2.2.5.3. Proteins were visualised with Coomassie brilliant blue R-250 and sized by comparison with a molecular weight marker (Mark12, NOVEX Australia).

4.2.2 Extraction of Rep protein

Cells from an IPTG-induced culture were harvested and resuspended in extraction buffer (50 mM NaH_2PO_4 pH 8.0, 300 mM NaCl, 10 mM β-mercaptoethanol). Cells were incubated with 1 mg ml^{-1} lysozyme on ice for 5 min, frozen in liquid nitrogen, thawed in cold water and then sonicated for 20 seconds. The lysates were centrifuged at 10000 *g* for 5 min at 4°C. The supernatant (soluble fraction) was transferred to a new tube and the pellet (insoluble fraction) was resuspended in extraction buffer. Both protein fractions were denatured and analysed in SDS-polyacrylamide gel as described in section 2.2.5.3.

To increase the level of protein solubilization under non-denaturing conditions, 10% glycerol and either 1% sarkosyl or 1% Tween 20 were added to the extraction buffer and Rep protein extracted as described above.

4.2.3 Purification of Rep protein under non-denaturing conditions

The soluble protein obtained from 250 ml of IPTG-induced culture was added to 4 ml of a 50% slurry of Ni^{2+}-NTA resin (QIAGEN), previously equilibrated with extraction buffer. The mixture was stirred at room temperature for 60 min and then loaded onto a 1.2 cm diameter column. After washing the column with 10 column volumes of extraction buffer, pH 8.0, supplemented with 20 mM imidazole and an additional 10% glycerol, the His-Rep fusion protein was eluted from the column by increasing the imidazole concentration to 250 mM. The eluate was concentrated 5 fold in 10 mM Tris-HCl, pH 8.0, 10% glycerol, 1 mM EDTA, 200 mM NaCl, 1 mM DTT and 1 mM phenylmethylsulfonyl fluoride (Thommes *et al.*, 1993) using Centricon concentrator columns (Amicon, USA). The concentrated protein was mixed with an equal volume of 100% glycerol and stored at -20°C. The concentration of the purified

His-Rep fusion protein was estimated by comparison to the known concentrations of bovine serum albumin using Coomassie brilliant blue R-250 staining following SDS-PAGE.

4.2.4 Electrophoretic mobility shift assay

A pair of TLCV-type oligonucleotide primers, $P169^c$ and $P2594^v$ (Table 2.2), was used to amplify a 342 bp fragment (Fig. 4.1, IR_T) containing the 298 bp intergenic region (IR). DNA amplification was performed as described (section 2.2.7). Digestion of the PCR product with *Eco* RI generated two fragments of 120 bp and 222 bp (Fig. 4.1, fragments IR_L and IR_R, respectively). Alternatively, the 120 bp fragment was amplified using the $P2718^c$ and $P2594^v$ primers (Table 2.2). Three other TLCV fragments referred to as fragments I, II and III (Fig. 4.1), were generated by double digestion of a TLCV clone by either *Xba* I and *Sac* I (387 bp), *Nco* I and *Xba* I (469 bp) or *Bam* HI and *Nde* I (383 bp), respectively. A full-length 356 bp DNA of the Darwin strain of potato spindle tuber viroid (PSTVd-D), released by *Bam* HI digestion of a PSTVd-D clone (see section 3.3.5), was used as a heterologous DNA in binding assays. A 177 bp PSTVd-D DNA fragment was amplified using primers $P91^c$ and $P271^v$ (Table 2.2).

PCR products or restriction fragments were subjected to electrophoresis in 1.2 or 1.5 % agarose gels in 1x TBE (Table 2.1), and the excised bands purified using a QIAquick gel extraction kit (QIAGEN). The DNA fragments (100 ng) were end-labelled using klenow fragment as described in section 2.2.15.2.

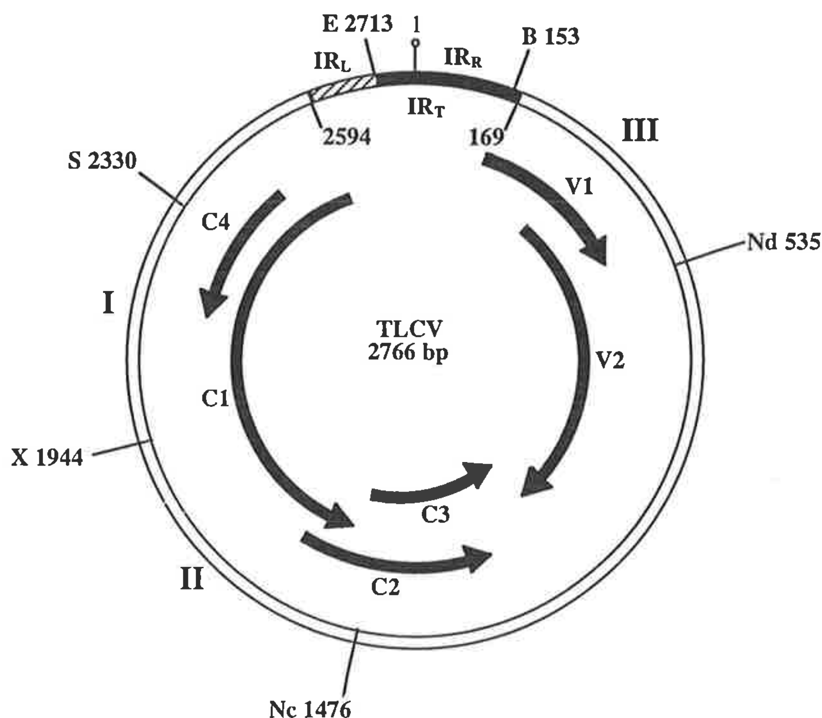


Fig. 4.1 *Genome organisation of TLCV.*

Functional ORFs on the virion-sense (V1 and V2) and the complementary-sense (C1, C2, C3, and C4) DNA strands are displayed by arrows. The position of the conserved stem-loop structure (⫯) is indicated. Fragments used in DNA-binding studies were IR_T (342 bp, containing the entire 298 bp intergenic region, nucleotides 2616- 147), IR_L (120 bp, hatched box), IR_R (222 bp, black box), and fragments I, II, and III. Restriction sites: B, Bam HI; E, Eco RI; Nc, Nco I; Nd, Nde I; S, Sac I; and X, Xba I. Numbering of the TLCV sequence is as in Dry et. al (1993).

Electrophoretic mobility shift assays were performed using the purified soluble His-Rep fusion protein. Typical binding reactions contained approximately 150 ng of His-Rep fusion protein, 1-2 ng labelled DNA and 1µg of either poly (dI-dC) or Salmon sperm DNA in 10 µl binding buffer (20 mM Hepes, pH 7.4, 40 mM KCl, 10% glycerol, and 1 mM DTT). Reactions were incubated at 25°C for 20 min and samples were

analysed by electrophoresis in non-denaturing 4 % polyacrylamide gels in 1x TBE (Table 2.1). The gels were dried on Whatman paper and radioactive bands visualised by autoradiography.

4.2.5 DNase I footprinting

The protocol for DNase I foot printing was essentially as described in Graves *et al.* (1986). TLCV P2718^{c} and P2594^{v} and PSTVd-D P271^{v} primers (100 ng) were 5′-end-labelled using T4 polynucleotide kinase as described in section 2.2.15.3. Each 5′-end-labelled primer was used directly without purification with an unlabelled primer in PCR reactions. The labelled DNA fragments were purified using a QIAquick PCR purification kit (QIAGEN).

His-Rep fusion protein (150 ng) was incubated with 1-2 ng ^{32}P-end-labelled DNA fragment in 10 μl of binding buffer containing 100 ng salmon sperm DNA for 20 min at 25°C. Control incubations contained all of the reaction components except for the His-Rep fusion protein. Reactions were diluted to 50 μl with water, supplemented first with 1 mM $MgCl_2$ and then with 5 U DNase I and incubated for 1 min at room temperature. The reaction was terminated by addition of an equal volume of stop solution (200 mM NaCl, 30 mM EDTA, 1% SDS) containing 10 μg *E. coli* tRNA (Boehringer Mannheim) as carrier. The reaction product was extracted once with an equal volume of phenol:chloroform (4:1) and once with an equal volume of chloroform:isoamylalcohol (24:1) and precipitated with two volumes of ethanol. The DNA fragments produced by partial DNase I digestion were resolved in an 8% sequencing gel in 1x TBE in parallel with sequencing reactions of the corresponding labelled DNA fragments. Sequencing was performed using a Sequenase kit (United States Biochemical) as described in section 2.2.14 and the same ^{32}P-labelled primers used for amplification of the fragments.

4.3 RESULTS

4.3.1 Expression and purification of Rep protein

Cloning of the C1 ORF of TLCV into the bacterial expression vector pQE30, in-frame with the 6xHis open reading frame, resulted in the production of significant amounts of His-Rep fusion protein after induction with IPTG (Fig. 4.2, compare lanes 1 and 2). The fusion protein migrated in SDS-containing gels to a position corresponding to an *M*r of approximately 44500 when compared to molecular weight markers. The *M*r of the fusion protein is slightly greater than that expected for the combined C1 translation product and vector-derived amino acids which is calculated to be 42469. Similar observations of higher than expected *M*r estimates have also been made for the AC2 protein of TGMV (Thommes and Buck, 1994) and of potato yellow mosaic geminivirus (Sung and Coutts, 1996) when expressed in *E. coli*.

The expressed His-Rep fusion protein was found almost entirely in the insoluble fraction of cell homogenates (Fig. 4.2, compare lanes 3 and 4). Attempts were made to solubilize the protein under non-denaturing conditions necessary for DNA-binding assays using either non-ionic detergent (Tween 20) or ionic detergent (sarkosyl). Lysing bacteria in extraction buffer containing 1% sarkosyl and 10% glycerol resulted in a high yield of soluble protein (results not shown). However, the solublized protein did not bind to the Ni-NTA resin used for affinity purification of the His-Rep fusion protein. In contrast, extraction in the presence of 1% Tween 20 and 10% glycerol produced a lower yield of soluble protein (Fig. 4.2 lanes 5 and 6), but the protein bound efficiently to the Ni-NTA resin allowing its purification to near homogeneity as judged by SDS-PAGE (Fig. 4.2 lane 7).

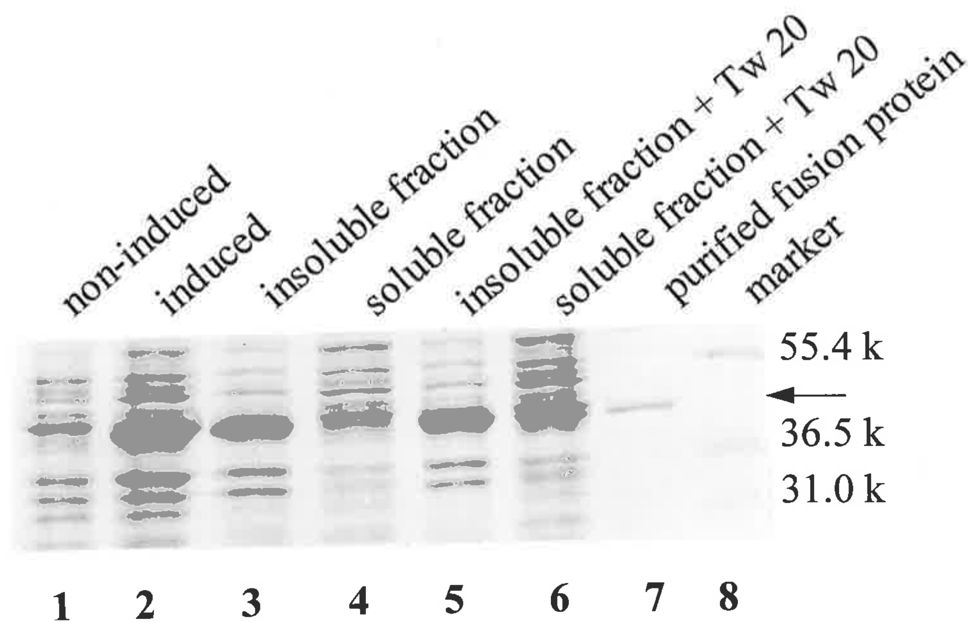


Fig. 4.2 *Analysis of the His-Rep fusion protein expressed in E. coli.*
E. coli strain M15 carrying pQE30-C1 was induced with 1 mM IPTG for 3 hours. Total protein was extracted and analysed by electrophoresis in a 10% SDS-polyacrylamide gel as described in Materials and Methods. Total E. coli protein extracts (lanes 1 and 2) or fractionated proteins (see below) were analysed. Insoluble and soluble protein fractions were extracted in the absence (lane 3 and 4) or presence (lanes 5 and 6) of 1% Tween 20 and 10 % glycerol. His-Rep fusion protein (lane 7) was purified by affinity chromatography using Ni^{2+}-NTA resin as described in Materials and Methods. The position of the His-Rep fusion protein is indicated by an arrow.

4.3.2 TLCV Rep binds specifically to the viral *ori*.

Incubation of ^{32}P-labelled dsDNA fragment of the TLCV IR_T with purified His-Rep fusion protein resulted in production of a discrete complex with retarded electrophoretic mobility (Fig. 4.3, lanes 1 and 2). Binding was drastically reduced when the same DNA fragment was heat-denatured to single-stranded form prior to incubation (data not shown), indicating a preference of Rep for interaction with dsDNA. An

optimum binding reaction contained approximately 150 ng of His-Rep fusion protein, 1.0 ng labelled DNA and 1.0 μg either poly (dI-dC) or salmon sperm DNA as heterologous competitor in 10 μl of binding buffer. The need for a high concentration of His-Rep fusion protein relative to target DNA concentration for optimal binding suggests that only a fraction of the purified Rep protein has the correct conformation to bind to the target DNA. Similar high ratio of protein to DNA concentrations have also been used in binding of C2 (AC2) protein, expressed in *E. coli*, of two other geminiviruses (Noris *et al*., 1996; Sung and Coutts, 1996).

The retarded complex was not present when the protein-DNA reaction product was incubated with 1.0 mg ml^{-1} proteinase K for 30 min at 37°C (Fig. 4.3, lane 11), demonstrating that the shift in probe mobility was protein-mediated. Furthermore, when His-Rep fusion protein was substituted with the same amount of bovine serum albumin in the binding reaction, no retarded complex was formed (data not shown) indicating the specificity of the binding reaction for the His-Rep fusion protein. The addition of divalent cations Mg^{2+}, Ca^{2+} or Zn^{2+}, each at 5 mM to the binding reaction prevented complex formation (results not shown), indicating that these cations induced unfavourable conformational changes to the protein and/or the probe. However, the complex was sufficiently stable in the presence of 1.0 mM Mg^{2+} which was required for DNase I activity in the footprinting experiments (see below).

The specificity of Rep-binding was examined using three different DNA fragments from the TLCV genome obtained from outside of the intergenic region (Fig. 4.1, fragments I, II and III) and from a DNA clone of PSTVd-D. His-Rep fusion protein failed to bind to any of these fragments (Fig 4.3, lanes 3-10) indicating the specificity of Rep for binding sequence(s) within the TLCV intergenic region.

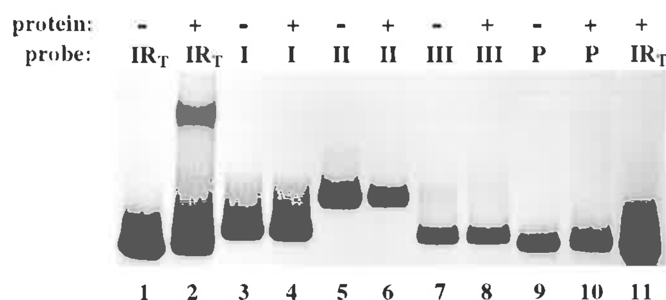


***Fig. 4.3** Electrophoretic mobility shift analysis of the interaction of Rep fusion protein with TLCV DNA fragments.*

Labelled DNA fragments of either the TLCV genome or a DNA clone of PSTVd-D were incubated in the absence (-) or presence (+) of His-Rep fusion protein. Reactions contained 1.0 µg of either poly (dI-dC) or salmon sperm DNA. Binding reactions were analysed by electrophoresis in a 4% polyacrylamide gel as described in Materials and Methods. The fragments IR_T (342 bp), I (387 bp), II (469 bp), and III (383 bp) were derived from TLCV (Fig. 4.1) and the fragment P was a full-length cloned DNA (356 bp) of the PSTVd-D (page 63). The binding reaction in lane 11 is the same as in lane 2, but was treated with proteinase K prior to electrophoresis.

Sequence specificity of the binding reaction was further tested by competition assays in which labelled TLCV IR_T dsDNA fragment was incubated in the presence of different molar ratios of unlabelled homologous or heterologous DNAs. The level of either poly (dI-dC) or salmon sperm DNA in competition assays was reduced from 1.0 µg to 100 ng per reaction as the level of competition was markedly improved in the presence of the lower level of DNA. Decreasing the concentration of poly (dI-dC) or

salmon sperm DNA from 1.0 μg to 100 ng in the binding reaction increased the number of retarded bands (compare lane 2 in Fig. 4.3 with lane 2 in Fig. 4.4) while complete absence of these heterologous DNAs shifted the labelled DNA fragment to the top of the gel (data not shown). The addition of a 150-fold excess of unlabelled homologous DNA to the binding reaction almost completely blocked complex formation with the labelled DNA fragment (Fig. 4.4, lane 3) whereas addition of the same amount of unlabelled heterologous PSTVd-D DNA had no significant effect on the formation of complexes of retarded mobility (Fig. 4.4, lane 4). These experiments further demonstrate that Rep interacts specifically with sequence(s) within the intergenic region of TLCV.

4.3.3 The stem loop structure is not essential for Rep-binding.

To delimit the Rep-binding site within the intergenic region, this region was subdivided into two fragments of 120 bp (IR_L) and 222 bp (IR_R), the latter containing the putative stem-loop structure (Fig. 4.1). Binding experiments using each of the two DNA fragments revealed that the electrophoretic mobility of the labelled 120 IR_L fragment, but not the labelled 222 bp IR_R fragment, was retarded by TLCV Rep (Fig. 4.5). These results indicated that the Rep-binding site maps to a region located between nucleotides 2594-2713 of the left side of intergenic region, and that the binding is not mediated by the putative stem-loop structure. However, it can be seen that the efficiency of binding of Rep to IR_L (Fig. 4.5, lane 2) was significantly lower than with IR_T (Fig. 4.4, lane 2). This apparent difference in binding efficiency suggests that IR_R may have a role in enhancing complex formation with TLCV Rep.

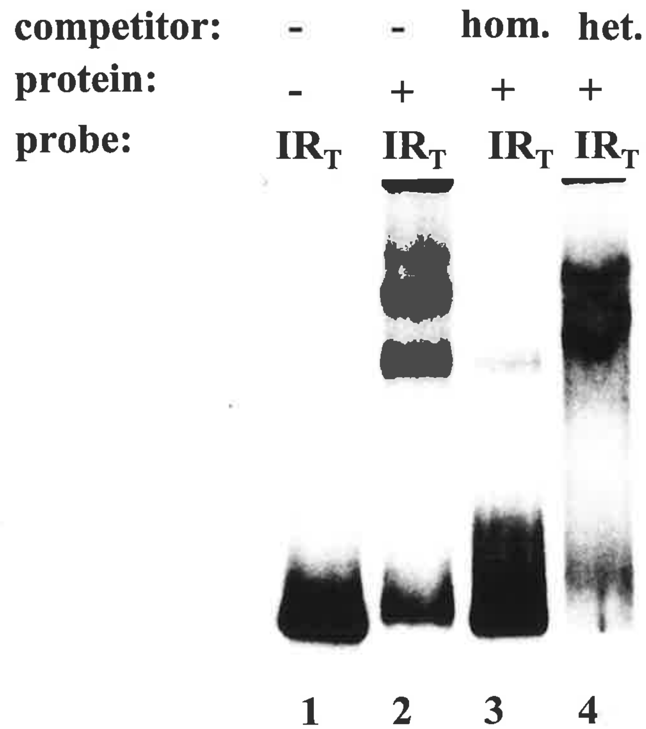


Fig. 4.4 *Effect of competitor DNAs on binding of Rep to the TLCV intergenic region. The labelled IR_T DNA fragment was incubated in the absence (-) or the presence (+) of His-Rep fusion protein. Binding reactions contained 100 ng of poly (dI-dC)* and *were carried out without competitor DNA (lane 2), with 150-fold excess of unlabelled homologous DNA (hom., lane 3) or with 150-fold excess of unlabelled heterologous PSTVd-D DNA (het., lane 4).*

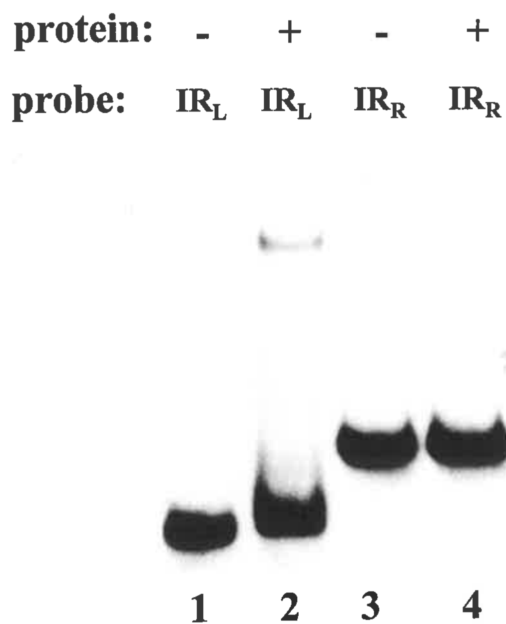


Fig. 4.5 *TLCV Rep binds specifically to the left part of intergenic region.*

Labelled DNA fragments of the TLCV 120 bp IR_L fragment (lanes 1 and 2) and the 222 bp IR_R fragment (lanes 3 and 4) were incubated in the absence (-) or the presence (+) of His-Rep fusion protein. Binding reactions were carried out and analysed as described in Fig. 3.

4.3.4 Determination of the nucleotide sequence of Rep-binding domains within the TLCV *ori*

The 120 bp IR_L fragment was labelled at the 5′ end of the virion sense DNA strand and used in DNase I footprinting. Two zones of the DNA fragment were consistently protected from DNase I digestion in the presence of TLCV Rep compared to control digestions (Fig. 4.6a). Comparison with the TLCV sequence revealed these two footprints (A and B) to be positioned 9 nucleotides apart in a region located between the TATA box and the initiation codon of the C1 (Rep) promoter (Fig. 4.6d). Footprint A includes the sequence GCAATTGGTGTCTCTCAA (nucleotides 2625-2642), while footprint B covered the sequence TGAATCGGTGTCTGGGG (nucleotides 2652-2668) and both contain a direct repeat of the motif GGTGTCT (underlined). When DNase I footprinting was carried out with the same 120 bp IR_L fragment carrying the ^{32}P label at the 5′-end of the complementary sense strand, only footprint B was observed (Fig. 4.6b). Footprint A was difficult to resolve unambiguously because certain residues in this region of the complementary-sense DNA probe were not digested by DNase I in control reactions carried out in the absence of Rep protein. No protected zones were observed when an unrelated PSTVd-D DNA fragment was end-labelled and used as a control in parallel reactions (Fig. 4.6c).

It is interesting to note that three bases, GTC, of the repeated element in footprints A and B were not protected when the footprinting was carried out with IR_L fragment labelled in the virion-sense strand (Fig. 4.6a) compared to complementary-sense strand (Fig. 4.6b). This observation suggests differential exposure of the two DNA strands within the Rep-binding complex to DNase I digestion.

Fig. 4.6 *Footprinting analysis of Rep-binding site within the TLCV ori.*

DNase I footprinting was carried out in the absence (-) and presence (+) of His-Rep fusion protein using: (a) 120 bp TLCV IR_L fragment, ^{32}P-labelled at the 5'-end of the virion-sense strand (lanes 5 and 6), (b) the same fragment, labelled at the 5'-end of the complementary-sense strand (lanes 5 and 6) and (c) 177 bp PSTVd fragment, ^{32} P-labelled at the 5'-end of the plus sense strand (lanes 1 and 2). TLCV-labelled fragments were generated by PCR with 5'-end-labelled primers $P2594^v$ and $P2718^c$ (Table 2.2). Dideoxy sequencing reactions (lanes 1-4 in a and b) carried out on the same fragments with the same 5'-end-labelled oligonucleotide primers were run on the same gel next to the footprinting assays in order to provide the sequence of the protected regions. The position of protected regions (footprints A and B) are indicated by bars and the TLCV sequence corresponding to these protected regions is shown in (d).

(d) Sequence of the 120 bp TLCV IR_L fragment. Numbering of the residues is as in Appendix A (page 128). The beginning of the C1 ORF and positions of primers $P2594^v$ and $P2718^c$ used to amplify the IR_L fragment are indicated by arrows. The sequence of footprints A and B and the putative TATA box of the C1 promoter are boxed. The direct repeat element (ggtgtct) conserved in both footprints is shown in lower case and the closely related inverted repeat sequence is underlined.

(a)

A C G T - +

footprint B

footprint A

1 2 3 4 5 6

TLCV IR_L
virion-sense strand labelled

(b)

A C G T - +

footprint B

1 2 3 4 5 6

TLCV IR_L
comp.-sense strand labelled

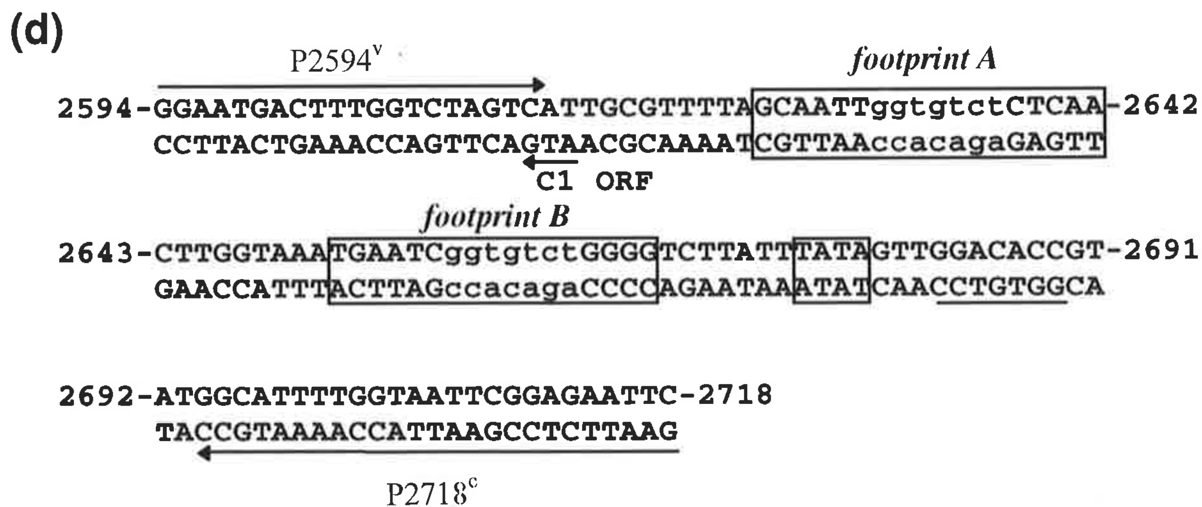
(c)

- +

1 2

PSTVd

(d)



4.4 DISCUSSION

To date, studies of Rep-binding elements in geminiviral *ori* have focussed on bipartite geminiviruses and have utilised Rep prepared by immunoprecipitation from plant or insect cell extracts (Fontes *et al.,* 1992; Fontes *et al*., 1994a). Using competitive DNA binding assays, Fontes *et al*., (1994a) were able to demonstrate that TGMV Rep binds specifically to a 13 bp element (GGTAGTAAGGTAG) located on the left side of the intergenic region between the transcription start site and the TATA box of the AC1 (Rep) promoter. However, direct characterisation of the Rep-binding domain by complementary methodologies such as mobility shift and footprinting assays was not possible due to potential interference from contaminating proteins immunoprecipitated from these crude cell extracts (Fontes *et al.,* 1992; Fontes *et al*., 1994a). In the present work, a bacterial expression system was used to produce Rep protein of a monopartite geminivirus. TLCV Rep was purified to homogeneity under non-denaturing conditions (Fig. 4.2) and used in both DNA-binding assays and DNase I footprinting to directly identify the sequence elements involved in Rep-binding within the TLCV *ori*.

Electrophoretic mobility shift assays demonstrated that TLCV Rep forms specific complexes with the intergenic region of TLCV (Fig. 4.3). The specificity of Rep-binding for the intergenic region is demonstrated by the fact that there was no binding observed with four heterologous DNA fragments, including three TLCV fragments derived from outside the intergenic region (Fig. 4.3). Furthermore, the ability of the His-Rep fusion protein to bind to the TLCV intergenic region was almost completely blocked by the addition of a 150-fold molar excess of unlabelled homologous competitor DNA, while the addition of a 150-fold molar excess of heterologous DNA had no significant effect on the pattern typically observed (Fig. 4.4). The direct

demonstration of specific Rep-binding to the intergenic region of the monopartite TLCV, using electrophoretic mobility shift assays, is also consistent with the results of studies with bipartite geminiviruses (Fontes, *et al.,* 1992; Orozco *et al.*, 1997; Thommes *et al.*, 1993).

Subdivision of the TLCV intergenic region into two fragments showed that the TLCV Rep-binding was targeted specifically to the 120 bp fragment covering the left-hand side of the intergenic region (Fig. 4.5). This confirms that the TLCV *cis*-acting sequences for Rep-binding are located in this part of the intergenic region as in other geminiviruses (Choi and Stenger, 1995; Fontes, *et al.,* 1992; Jupin *et al.*, 1995; Lazarowitz *et al.*, 1992).

Further delimitation of the TLCV Rep-binding sequence elements within the left-hand side of the intergenic region was achieved by DNase I footprinting. Footprinting of the 120 bp IR_L fragment indicated the presence of two protected regions in the presence of TLCV Rep (Fig. 4.6). Each footprint is approximately 17-18 nucleotides in size and both contain the direct repeat element GGTGTCT as a core sequence. A closely related sequence element (GGTGTCC) is also located in an inverted orientation downstream of the TLCV C1 promoter TATA box (Fig. 4.6d nucleotides 2683-2689). However, there was no evidence of any protection of this sequence in our footprinting experiments, indicating that TLCV specific Rep-binding domains, within the viral *ori*, are confined to regions A and B (Fig. 4.6).

The observed protection of sequences bordering the TLCV repeat motifs in footprints A and B (Fig. 4.6) suggests that Rep-binding may not be limited to repeat elements. However, the possibility that the protection of the bordering sequences results from exclusion of DNase I due to the formation of the DNA-Rep complex involving

only the repeat elements cannot be ruled out. Alternative methods of DNA footprinting analysis, using smaller chemical cleavage agents (Papavassiliou, 1994; Ragnhildstveit *et al.*, 1997), would help to resolve this question.

The GGTGTCT repeat element has previously been postulated by Argüello-Astorga *et al.* (1994) to be a Rep specific-binding site. Fontes and co-workers (1994a; 1994b) have established that the recognition sequence required for Rep-binding to the cognate intergenic region of the bipartite geminiviruses TGMV and BGMV is a 12-13 bp sequence element containing the direct repeat elements GGTAG (TGMV) and TGGAG (BGMV). The results presented here with the monopartite TLCV further support the suggestions that these direct repeats function as core elements in Rep recognition and binding and that the sequence of the repeat element is specific for each geminiviral Rep.

The location of the Rep-binding elements between the TATA box and the initiation codon of the C1 (Rep) ORF appears to be highly conserved between the subgroup III viruses TGMV (Fontes *et al.*, 1994a), BGMV (Fontes *et al.*, 1994b) and TLCV (Fig. 4.6). This is also consistent with the observed Rep-mediated repression of C1 gene expression (Eagle, *et al.,* 1994; Haley *et al.*, 1992; Sunter, *et al.,* 1993), as Rep binding at this position would interfere with transcription of the C1 ORF by RNA polymerase. However, the relative positioning of the two repeats appears to vary significantly between each of the two evolutionary branches of the subgroup III viruses (Argüello-Astorga *et al.*, 1994). For example, the distance between these repeat elements in BGMV and TGMV, both New World geminiviruses, is 2 and 3 nucleotides respectively, while the repeat elements in TLCV, an Old World virus, are 20 nucleotides apart. The significance of this variable spacing on Rep-binding remains unknown.

The presence of two retarded bands observed in electrophoretic mobility shift assay experiments (Fig. 4.4, lane 2) may indicate the possible binding of more than one Rep molecule to the TLCV intergenic region. This possibility is consistent with the recent findings that geminiviral Rep contains a domain specifying protein oligomerisation and that multimerisation may be a prerequisite for DNA binding (Orozco *et al.*, 1997).

The relative functions of the individual footprints A and B in TLCV replication remain unknown. In TGMV, two sequence elements have been shown by mutagenesis to be involved in Rep-binding and viral DNA replication (Fontes *et al.*, 1994a). However, the existence of two Rep-binding domains within the TLCV *ori* contrasts with the presence of only a single putative Rep-binding sequence element in the recently described TLCV-satellite DNA (Dry *et al.*, 1997). The 682 nt ssDNA satellite depends on TLCV for replication and has no significant sequence similarity to the TLCV helper virus genome other than the conserved TAATATTAC motif, found in all geminiviruses and the sequence element TGAATCGGTGTCT which is also present in footprint B (Fig. 4.6). Further analysis of the interaction of TLCV Rep with the satellite genome by electrophoretic mobility shift assay and DNase I footprinting will reveal the role of this single binding domain in TLCV Rep-mediated satellite replication (Dry *et al.*, 1997).

Chapter Five

Strain-Specific Determinants of Tomato Leaf Curl Geminivirus DNA Replication

5.1 INTRODUCTION

Phenotypic and genetic analyses have revealed a surprising degree of variability among geminivirus strains. Choi and Stenger (1995) demonstrated that DNA replication factors of two strains of BCTV are not functionally interchangeable. They raised the question as to whether the two BCTV strains they studied should be considered as distinct viruses or as distinct types of the same virus. Similarly, Gilbertson *et al.* (1993a) and Faria *et al.* (1994) designated certain strains of BGMV as type I or type II categories based on pseudo-recombination experiments between BGMV cloned DNAs and major differences in DNA sequence and biological properties (e.g., sap-transmissibility, symptomatology and host range). It was of interest, therefore, to examine whether viral functions are interchangeable between the strains of TLCV.

In Chapter Four the identification of the Rep binding sequences within the TLCV *ori* was reported. Here, using *in vivo* complementation tests and *in vitro* Rep binding assays, it is demonstrated that replication functions are not interchangeable between the closely related TLCV strains.

5.2 MATERIALS AND METHODS

5.2.1 Cloning

An infectious clone of TLCV D1 was produced using primers from a region containing a unique *Kpn* I site. The oligonucleotide primers, P1588^c and P1583^v (Table 2.2), containing an overlapping *Kpn* I site were used to clone a DNA monomer of D1 into pBluescript SK$^+$ (see also section 3.2.3). A dimeric (head-to-tail) clone was obtained directly using a 10:1 molar ratio of insert to vector in the ligation reaction. The identity of the dimeric clone was confirmed by restriction analysis. The dimeric construct was cloned in the binary transformation vector pBin19 (Bevan, 1984) and introduced into *A.*

tumefaciens strain C58 by electroporation (section 2.2.11).

An infectious clone of the TLCV type strain was from Dry *et al.* (1993) and a head to tail 1.5-mer of type C1 mutant (C1mut) was from Rigden *et al.* (1993). A C2 mutant of the TLCV type strain, supplied by Dr I. B. Dry, was produced by the introduction of 2 nucleotides (AA) at nucleotide position 1525 causing the formation of a premature TAA stop codon within the C2 ORF. This mutation also produced a second *Vsp* I restriction site in addition to an existing site at nucleotide position 1075. A 1.5-mer C2 mutant (C2mut) construct was produced in pBin19 as described in Rigden *et al.* (1996).

5.2.2 Infectivity assays

Infectivity experiments were conducted by agroinoculation of tomato and datura plants. Agroinoculation was performed with a 48-72 h culture of *A. tumefaciens* containing tandem repeat constructs in pBin19 (Dry *et al.*, 1993). Bacterial cultures bearing TLCV constructs were inoculated singly or mixed in equal proportions. Developing leaves were sampled 21-28 days post-inoculation, total nucleic acids were extracted as described (section 2.2.3) and the presence of viral DNA was tested by PCR using specific primers to either TLCV type ($P2594^v$, Table 2.2) or TLCV D1 strain ($P2639^c$, Table 2.2) together with a non-specific primer. The presence of DNA from C2 mutant progeny in the PCR product of plants co-infected with TLCV C1 and C2 mutants was verified by the digestion with *Vsp* I. The presence of the C1 mutation was confirmed by nucleotide sequencing of the mutated region.

5.2.3 Electrophoretic mobility shift and DNase I foot printing assays

DNA fragments (120 bp) of the left part of the IR were generated by PCR (section 2.2.7) using the primers $P2718^c$ and $P2593^v$ identical to the D1 strain sequence or $P2718^c$ and $P2594^v$ identical to the type strain sequence (Table 2.2). These fragments

were ^{32}P-end-labelled as described (section 4.2.5) and used in electrophoretic mobility shift assays and footprinting using purified Rep protein of TLCV-type strain (section 4.2.3) as described in sections 4.2.4 and 4.2.5, respectively.

5.3 RESULTS

5.3.1 Infectivity of TLCV constructs

Agroinoculation experiments with TLCV-type clones, giving rise to disease symptoms indistinguishable from those of the field isolate, have previously been described (Dry *et al.*, 1993). To test the infectivity of a dimeric clone of TLCV D1, 10 tomato and 10 datura plants were agroinoculated with bacterial suspension containing this construct. Symptoms typical of tomato leaf curl disease were observed on newly developed leaves of all plants 21-28 days after inoculation. The onset of symptoms was approximately one week later compared to plants agroinoculated with the TLCV type strain. The D1 strain also induced milder symptoms compared to the type strain (Fig. 5.1) as had been observed in graft-inoculated plants from field sources.

Fig. 5.1 *Mild symptom of TLCV D1 strain (right) compared to severe symptoms of type strain (left) on agroinoculated tomato plants.*

5.3.2 Complementation experiments

In spite of 95% overall identity between the overall sequence of the TLCV type and D1 strains, the differences in the sequences of the intergenic region and the N-terminal portion of the Rep protein (see section 3.3.4) raised questions as to whether the D1 and TLCV type strain could complement each other for replication. In order to conduct a complementation assay, it was first examined whether co-inoculation with two separate virus strains leads to mixed infection. The existence of each virus strain in co-agroinoculated plants was demonstrated by PCR using primers specific to either the TLCV type or D1 strains. The PCR products of each strain were further distinguished by restriction enzyme analysis using *Sca* I, a restriction endonuclease that digested only D1 DNA, and *Afl* III, which digested only type strain DNA. The results showed that the TLCV type and D1 strains can replicate together within a single co-agroinoculated plant (Table 5.1, row 3).

In the next experiment, 3 tomato and 3 datura plants were inoculated with the D1 strain and a replication deficient C1mut of the type strain. The co-agroinoculated plants showed mild disease symptoms and contained only the D1 strain (Table 5.1, row 5) indicating that TLCV D1 could not complement a C1 mutant of the type strain.

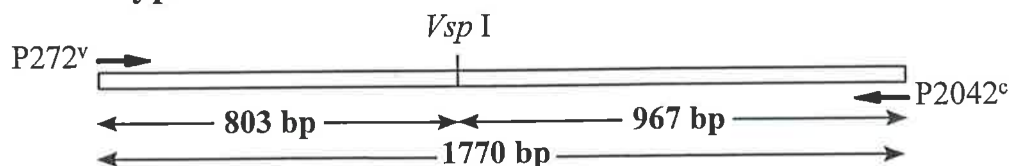
To ensure that the observed lack of complementation was not due to an unidentified defect in the type strain C1mut construct, this construct was co-agroinoculated with a C2mut construct of the same strain in both tomato and datura plants. Neither the C1mut nor the C2mut were infectious individually (Table 5.1, rows 4 and 6). However, a mixture of the two mutants produced infectivity in all 6 tomato and all 4 datura plants tested (Table 5.1, row 7).

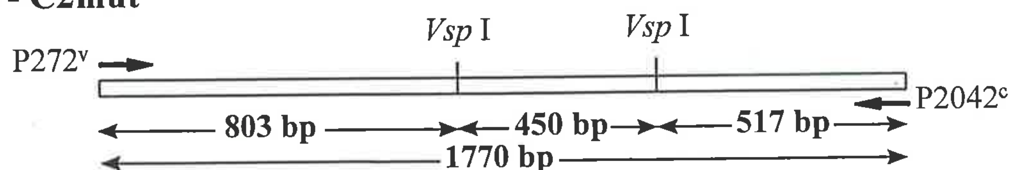
Table 5.1 *Detection of TLCV strains in agroinoculated plants by PCR.*

	Inoculum	DNA species detected		
		primers specific to type strain	primers specific to D1 strain	non-specific TLCV primers
1	type strain	+	-	+
2	D1 strain	-	+	+
3	D1 + type strain	+	+	+
4	C1 mutant[a]	-	-	-
5	D1 strain + C1 mutant	-	+	+
6	C2 mutant[a]	-	-	-
7	C1 mutant + C2 mutant	+	-	+

[a]C1 and C2 are mutants of TLCV type strain.

To confirm that the infection by C1mut plus C2mut resulted from complementation and not recombination, diagnostic tests were carried out on the progeny DNAs. A set of TLCV type primers (P272[v] and P2042[c]) was used to amplify a 1770 bp fragment from either C1mut + C2mut co-infected plants or from the TLCV-type infected plants. *Vsp* I digestion of the PCR product obtained from the C1mut + C2mut co-infected plant (Fig. 5.2C, lanes 3 and 4) produced, in addition to 803 bp and 967 bp fragments, a small portion of 517 bp and 450 bp fragments (resulted from digestion of 967 bp fragment) which is diagnostic of the C2mut construct having two *Vsp* I sites (Fig. 5.2B). This is in contrast to DNA amplified from the TLCV type infected plants which contained only a single *Vsp* I site (Fig. 5.2A) and produced 803 bp and 967 bp fragments when digested by *Vsp* I (Fig. 5.2C, lanes 1 and 2).





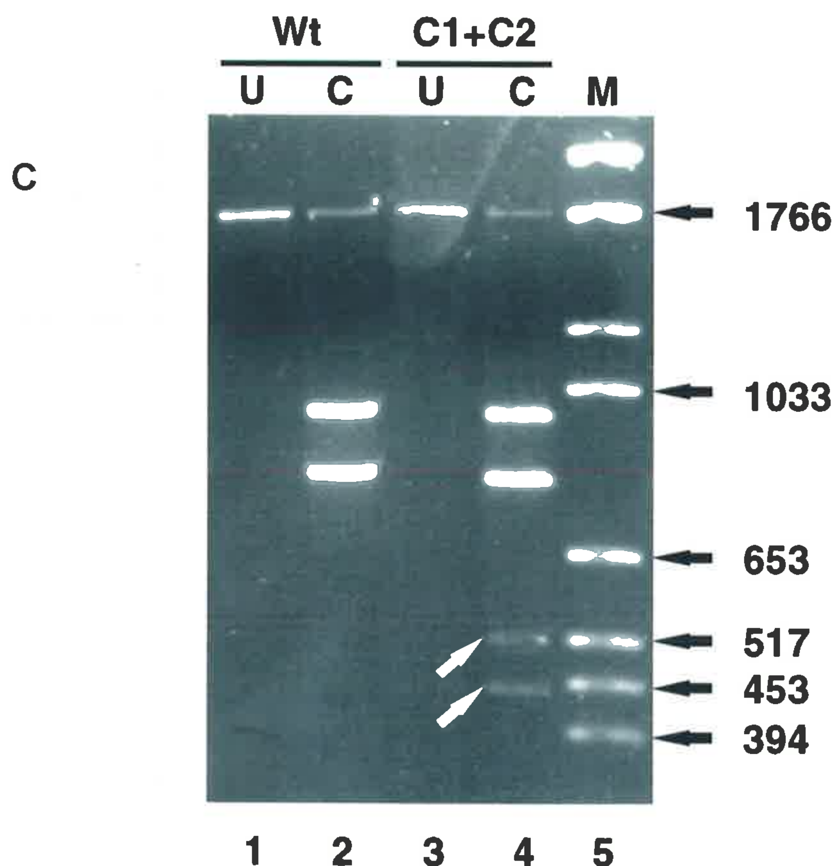


Fig. 5.2 A & B) DNA fragments resulting from Vsp I digestion of PCR products of wild-type or C1mut (A) and C2mut (B) genomes. C) PCR products amplified from plants infected with wild type TLCV (lanes 1 and 2) or with co-infected C1mut + C2mut (lanes 3 and 4). The DNAs are either uncut (U, lanes 1 and 3) or cut (C) by Vsp I (lanes 2 and 4).

The relative intensities of the components of the PCR product (Fig. 5.2C, lanes 3 and 4) having either one (C1mut DNA) or two *Vsp* I sites (C2mut DNA) suggested that the major proportion of TLCV DNA in C1mut + C2mut co-infected plants was derived from the C1mut construct. To confirm that the C1 mutation was still present, a PCR fragment obtained from the extract of the same C1mut + C2mut co-infected plant was sequenced across the position of the C1 mutation. Fig, 5.3 shows that the introduced mutation at the initiation start site of the C1 ORF represents the predominant DNA sequence present in this PCR mixture. Thus, the presence of both C1 and C2 mutant DNA in the mixed inoculation plants indicates that the two defective genomes must have complemented each other for the respective gene products.

Taken together, these observations suggest that, in spite of a high degree of sequence homology, the TLCV D1 can not support the replication of a C1 mutant of the type strain.

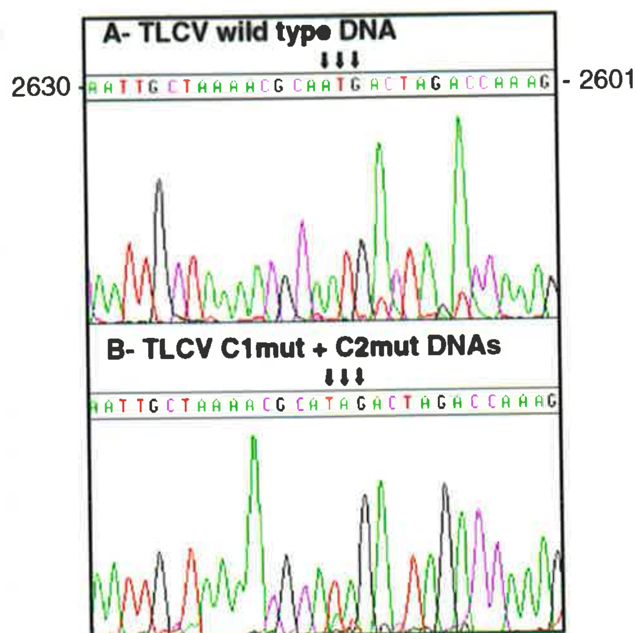


Fig. 5.3. *TLCV sequencing data of (A)wild type DNA and (B) mutant DNA from C1mut + C2mut co-inoculated plant. The ATG initiation codon of C1 ORF at positions 2613-2615 of wild type DNA (A) that has been altered to a TAG termination codon in C1mut DNA (B) are indicated by arrows. The wild type sequence contributed by the presence of the C2mut DNA can be seen as the minor peaks in (B).*

5.3.3 TLCV DNA Rep-binding domains confer replication specificity.

As discussed in Chapter Four the purified Rep protein of the TLCV type strain specifically binds to a 120 bp fragment in the 5′-region of the IR causing gel mobility retardation (Fig. 5.4, lanes 1 and 2). When the equivalent DNA fragment from the D1 strain was incubated with TLCV type strain Rep, no complexes were formed (Fig. 5.4, lanes 3 and 4) indicating that the Rep protein of the type strain can not recognise the binding domains within the IR of the D1 strain.

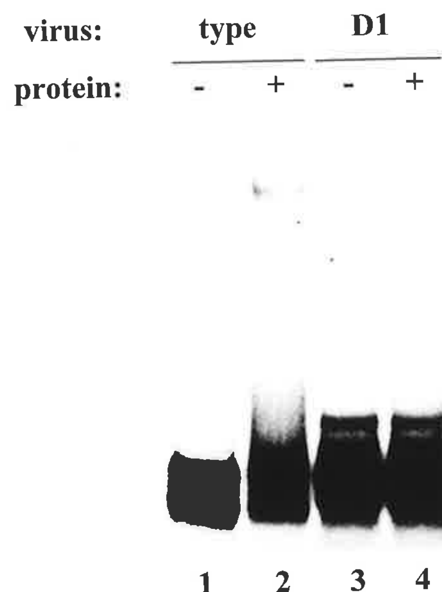


Fig. 5.4 *Electrophoretic mobility shift analysis of the interaction of TLCV type Rep with fragments of the left part of the intergenic region of type and D1 DNAs.*

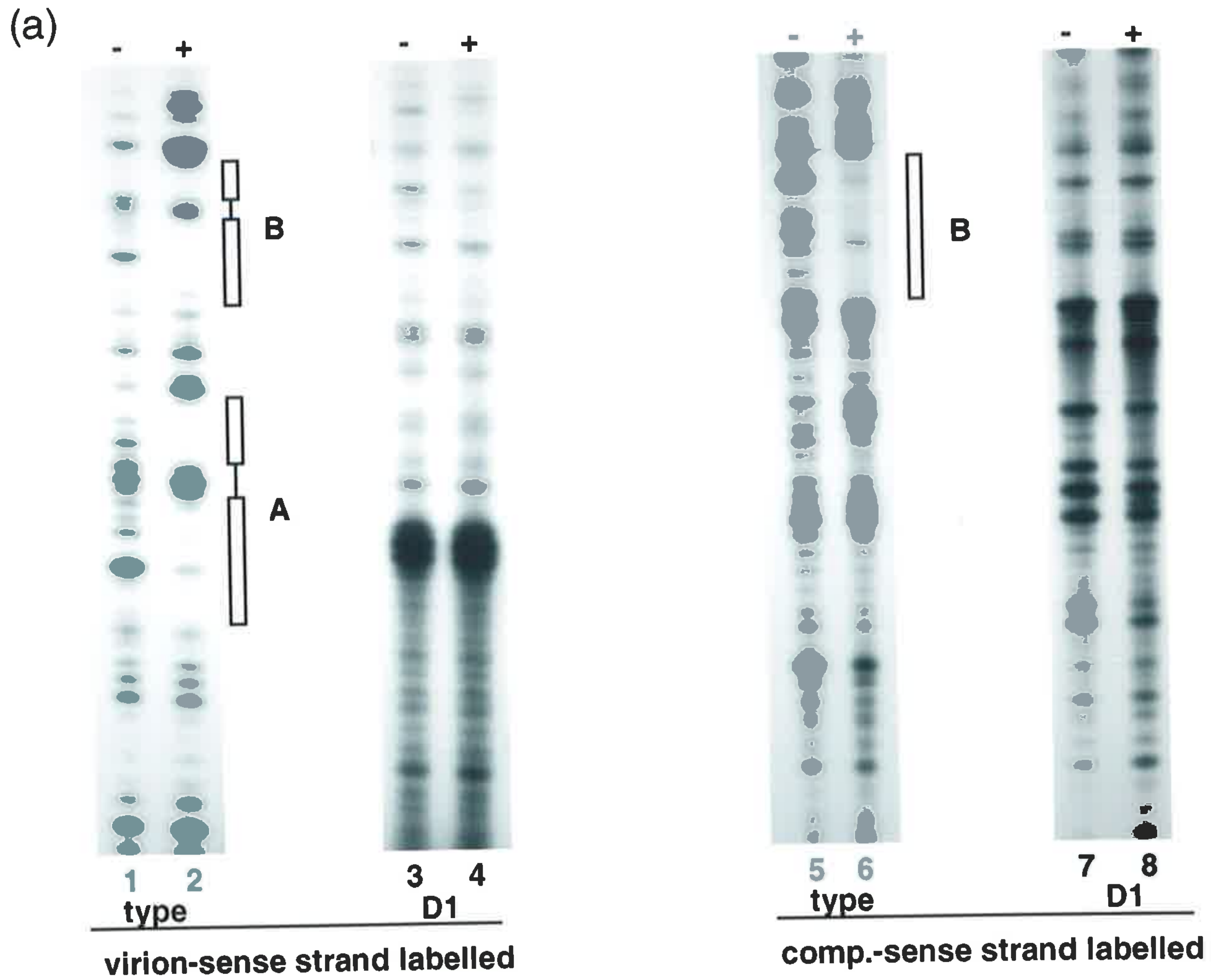
Labelled 120 bp fragments of either the TLCV type strain (lanes 1 and 2) or D1 strain (lanes 3 and 4) were incubated in the absence (-) or the presence (+) of His-Rep fusion protein and analysed in a 4% polyacrylamide gel.

DNase I footprinting of the 120 bp fragment of D1 strain also did not reveal any protected zones of this fragment (Fig. 5.5a, lanes 4 and 8), whereas two sequence elements of the same fragment of the type strain were protected from digestion by DNase I in the presence of type strain Rep (see section 4.3.4 and Fig. 5.5a, lanes 2 and 6). These results confirmed that the type strain Rep does not bind to D1 strain DNA and are consistent with the results of the *in vivo* complementation experiments whereby D1 DNA can not complement a defect in the type C1 construct.

Fig. 5.5. *DNase I footprinting analysis of Rep-binding sites within the ori of TLCV strains.*

(a) DNase I foot printing of the 120 bp fragment of the TLCV type strain (lanes 1, 2, 5, and 6) or the TLCV D1 strain (lanes 3, 4, 7 and 8) ^{32}*P-labelled at the 5'-end of the virion-sense strand (lanes 1-4) or at the 5'-end of the complementary-sense strand (lanes 5-8). DNA probes incubated in the absence (-) or in the presence (+) of the type Rep. The position of protected regions (footprints A and B) are indicated by bars. Samples were analysed by electrophoresis in a 8% polyacrylamide gel containing 8 M urea in 1x TBE.*

(b) Sequence alignment of the 120 bp DNA fragments of the TLCV type and D1 strains. Numbering of the residues as in Dry et al . (1993). The beginning of the C1 ORF is indicated by arrow. The sequence of footprints A and B of the type strain DNA containing the direct repeat element (ggtgtct) are boxed. Dots indicate conserved nucleotides in the sequence of the D1 strain relative to the type strain.



(b)

```
                2594                                                  2642
Strains         |                    C1 ORF             footprint A      |
Type            GGAATGACTTTGGTCTAGTCATTGC-GTTTTA[GCAATTggtgtctCTCAA]
D1              TA..ACG...A..GGGT.C...GT.A.CAAA.TG......A.A.AA....

                2643                                                  2691
                 |              footprint B                              |
Type            -CTTGGTAAA[TGAATCggtgtctGGGG]TCTTATTTATAGTTGGACACCGT
D1              A....C.........T..A.A....A..ACA..A.....G..-T.T..AA

                2692                       2718
                |                          |
Type            ATGGCATTTTGGTAATTCGGAGAATTC
D1              .................T.........
```

5.4 DISCUSSION

The biological relatedness of the wild type and D1 TLCV strains (Chapter Three) was studied using *in vivo* complementation experiments. The D1 and type strains could replicate simultaneously within the same host, but the D1 strain could not complement a replication-defective mutant of the type strain. This demonstrated that each strain replicated independently in co-infected plants.

The inability of the D1 strain to complement the C1 mutant of the type strain *in vivo* was consistent with the inability of the type strain Rep to bind to the D1 IR *in vitro*. These experiments demonstrated that the Rep-binding domains within the IR of the type strain (footprints A and B, Fig. 5.5) are responsible for strain-specific interaction with the cognate Rep molecule. The corresponding nucleotide sequences of the A and B regions within the D1 strain are ATGAATTGGAGACAATCA and AATTGGAGACTGGGG, which differ in six nucleotide residues in region A and four nucleotide residues in region B from the TLCV type strain (Fig. 5.5b), respectively. The direct repeat element GGTGTCT of the TLCV type strain is replaced by the motif GGAGAC(A/T) in the TLCV D1 strain.

These repeat sequences may function as core elements in TLCV Rep:DNA binding, governing strain-specific replication. However, sequences outside of the repeated elements may also be involved in Rep-binding and strain specificity. These observations support the results reported by Choi and Stenger (1996), who mapped the strain-specific *cis*-acting element of BCTV DNA replication to the directly repeated motifs in the *ori*. The Rep of CHF and Logan strains could recognise and replicate a chimeric BCTV genome containing a heterologous *ori* so long as all or portions of the core element of the directly repeated motif were derived from the same strain as the Rep

protein.

In bipartite geminiviruses, the direct repeat elements are identical between DNA A and B components. The Rep encoded by the A component can bind to B component, resulting in the replication of B component. Complementation studies conducted so far between different geminiviruses indicate that viable pseudorecombinants have only been observed between very closely related viruses or strains of a virus with identical Rep:DNA binding domains (Faria *et al.*, 1994; Gilbertson *et al.*, 1993b; Lazarowitz *et al.*, 1992). The A component cannot *trans*-replicate a heterologous B component if the latter contains different A and B Rep binding sites (Fontes *et al.*, 1994b; Frischmuth *et al.*, 1993; Lazarowitz *et al.*, 1992). These results support the view that specific interaction between a Rep protein and its cognate genome confers geminivirus replication specificity.

Using site-directed mutagenesis and competition assays, Fontes *et al.* (1994a) demonstrated the importance of the conserved GG dinucleotides of a direct repeat element for TGMV AC1:DNA binding. In their study, no replication was detected when DNAs contained mutations in GG dinucleotides in either of the repeat motifs of the AC1 recognition sequence. In these experiments, the AC1 function was provided in *trans* from a plant gene expression vector.

Comparisons of repeat elements of binding sites of two TLCV strains (Fig. 5.5b) also revealed that GG dinucleotides are conserved in the repeat elements of the two strains. However, the T residues of the type strain repeat element occur as an A residue in the D1 repeat element, suggesting that nucleotides other than GG are also important in determining the potential interaction between Rep and the binding domains. The

significance each individual nucleotide in determining the specificity of the Rep binding domain needs to be further investigated by mutational analyses.

Chapter Six

Subgenomic DNAs Associated with TLCV Replication

6.1 INTRODUCTION

In addition to the full-length geminiviral DNA genome, smaller virus-specific DNA molecules have been reported in plants infected with different geminiviruses, including ACMV (Stanley and Townsend, 1985), BCTV (Frischmuth and Stanley, 1992; Stenger *et al*., 1992), TGMV (Hamilton *et al.,* 1982; MacDowell *et al.,* 1986), TYLCV (Czosnek *et al*., 1989) and WDV (MacDonald *et al.,* 1988). These subgenomic viral DNAs are generally about half the size of the standard viral genome (MacDonald *et al.,* 1988; MacDowell *et al.,* 1986; Stanley and Townsend, 1985), but in BCTV, subgenomic DNAs ranging in size from 0.8 to 1.8 kb have been observed (Stenger *et al*., 1992). In bipartite geminiviruses, the subgenomic DNAs are usually derived from the DNA B component.

Inoculation of plants with cloned genomic DNAs results in a wild-type infection, usually without production of subgenomic DNA in the inoculated plants. However, subsequent passage of progeny virus may lead to the appearance of subgenomic DNAs (Czosnek *et al*., 1989; MacDonald *et al.,* 1988; MacDowell *et al.,* 1986; Stanley and Townsend, 1985). Unlike other geminiviruses, BCTV subgenomic DNAs appear upon infection of plants with cloned DNA inocula (Frischmuth and Stanley, 1992; Stenger *et al*., 1992).

In this chapter, characteristics of subgenomic DNAs associated with TLCV infection are reported and their dependence on the virus for encapsidation and replication are described.

6.2 MATERIALS AND METHODS

6.2.1 Plant material

The source of plant material used in the work described in this chapter was a single tomato plant, designated Darwin 3 (D3), grafted with a TLCV-infected tomato scion collected from a field near Darwin.

6.2.2 Construction of clones

Dimeric (head-to-tail) clones of subgenomic DNAs were constructed and transformed into *A. tumefaciens* as described in sections 2.2.11 and 5.2.1. TLCV type strain constructs containing tandem repeat DNAs of a full-length genome (Dry *et al.*, 1993) and a V1/V2 mutant with a 510 bp deletion (Rigden *et al.*, 1993) were also used.

6.2.3 Whole plant infectivity assays

Datura, tomato and *N. benthamiana* were inoculated with an agrobacterial culture containing a TLCV DNA construct (Dry *et al.*, 1993). Developing leaves were sampled 21-28 days post-inoculation, DNAs extracted as described in Section 2.2.3 and analysed by Southern blot hybridisation (Section 2.2.16).

6.2.4 Transient leaf-strip assay

The transient replication assay was performed as described by Dry *et al.* (1997). Leaf strips (1-2 mm) were cut from *in vitro* grown tobacco plants (*Nicotiana tabacum* cv. Samsun) and inoculated by placing them for 5 min in a culture of *A. tumefaciens* containing viral constructs. The strips were maintained on 0.7% bacto-agar plates containing culture medium (0.43% MS salts, 3% sucrose, 0.01% Gamborg vitamins) in the dark at 25°C for 48 h. They were then transferred to 25 ml of the same culture medium supplemented with 0.025% kanamycin and 0.05% cefotaxime (to kill *A. tumefaciens*) and incubated for 6 days at 25°C in normal room light with agitation at 50

rpm. Strips (150 mg) were blotted dry, frozen in liquid nitrogen and used for DNA extraction (section 2.2.3).

6.2.5 Immunocapture PCR

Immunocapture was carried out essentially as described by Wetzel *et al.* (1992). Plant leaf tissues were extracted in 10 volume of 50 mM Tris-HCl (pH 8.0), 5 mM EDTA, 2% polyvinylpyrolidone and 0.05% Tween-20 and centrifuged at 13,000 *g* for 15 min at 4°C. To capture TLCV particles, a polyclonal antibody against the coat protein of the serologically-related ACMV (Thomas *et al.,* 1986) was used. The subsequent steps are outlined in Table 6.1.

Table 6.1 *Immunocapture PCR*

1. Coat microplates with antibody (diluted 2000 fold in coating buffer) for 2 h at room temperature (100 μl/well).
2. Rinse 3 times briefly and wash once for 3 min with PBS-Tween 20.
3. Place 100 μl of plant and/or DNA extract per well, incubate at 4°C overnight.
4. Wash 3 times for 3 min with PBS-Tween 20.
5. Add 20 μl of 1% (v/v)Triton X100 solution per well.
6. Heat at 65°C, 5 min.
7. Transfer the resulting solution to fresh microcentrifuge tubes
8. Use 1 ul of solution for PCR amplification (Section 2.2.7)

6.3 RESULTS

6.3.1 Detection of TLCV subgenomic DNAs

Southern blot analysis of DNA extracted from the D3 tomato plant using a full-length TLCV probe revealed the presence of two smaller viral DNA bands in addition to the viral full-length ssDNA (Fig. 6.1A lane 1). These subgenomic (SG) DNAs were

identified as single-stranded by blotting in the absence of a denaturation step and hybridising with the same TLCV probe (Fig. 6.1B, lane 1). In the absence of denaturation, dsDNA does not hybridise to the probe.

The presence of SG DNAs was also examined in agroinfected plants. Southern blot analysis of DNA extracts of 3 datura and 3 *N. benthamiana* plants demonstrated that SG DNAs were produced in all plants agroinfected with a wild type TLCV construct. The size of the SG DNAs in datura was different from that of *N. benthamiana* (Fig. 6.1A, compare lanes 3 and 4), but appeared the same in individual plants of each species. The timing of their appearance in individual plants varied between 5 to 12 weeks after inoculation. Southern blot analysis of DNA extracts of a single agroinfected datura plant at different postinoculation dates is shown in Fig. 6.1C indicating the appearance of SG DNA 12 weeks postinoculation.

No SG DNA was produced in any of 6 TLCV-infected tomato plants tested (Fig. 6.1A lane 2) even one year after inoculation. Scions from TLCV-infected datura plants containing SG DNAs were grafted onto healthy tomato plants. Three weeks after grafting, the plants became infected and contained the same SG DNA as in the scion (Fig. 6.1D). These observations demonstrated that tomato is a suitable host for the accumulation of SG DNAs but does not support the *de novo* production of SG DNAs under the experimental conditions used.

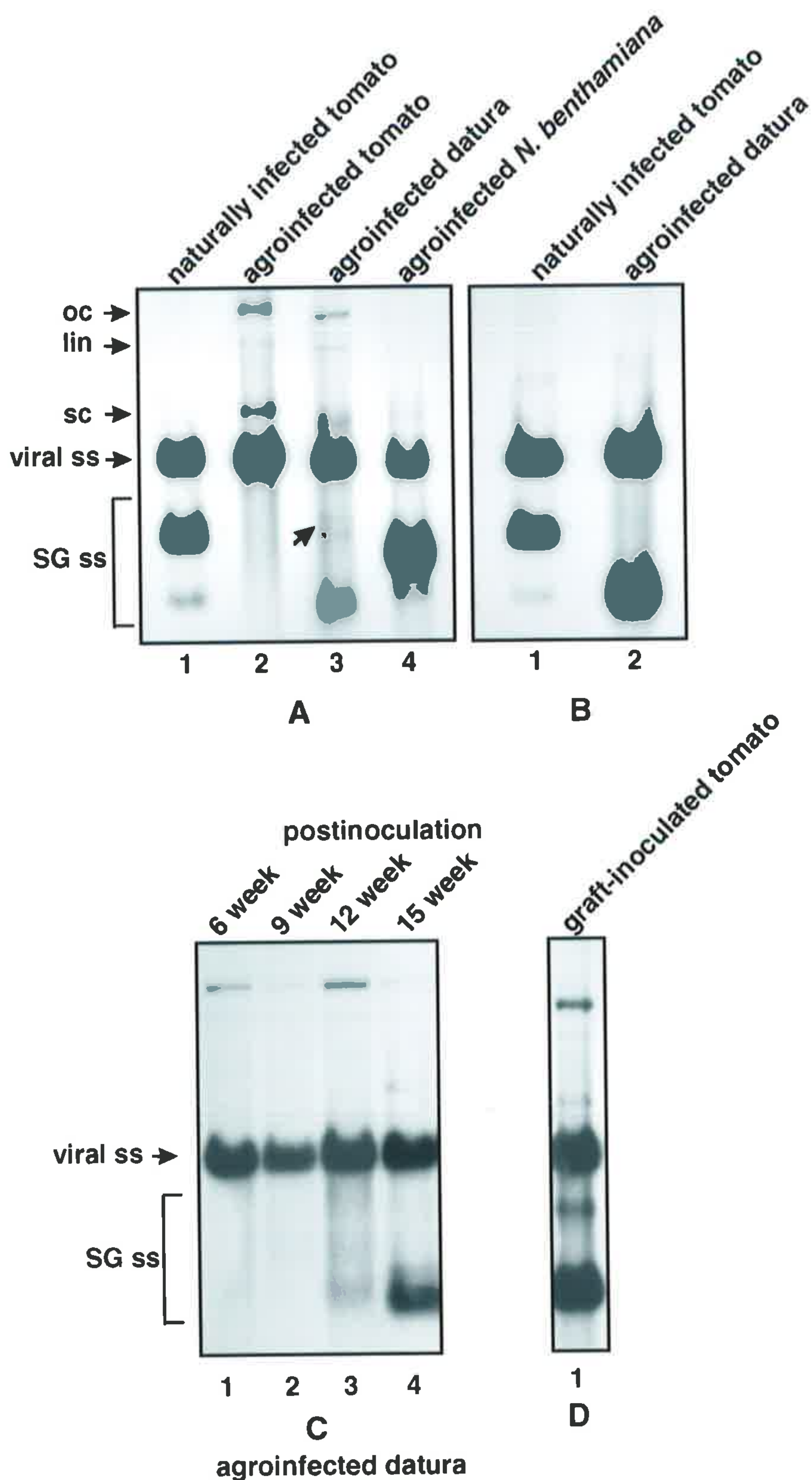


Fig 6.1 *Presence of subgenomic DNAs in TLCV-infected plants.*

A, C & D) Southern blotting under denaturing conditions with 0.4M sodium hydroxide. B) Blotting under non-denaturing conditions with 10x SSC. DNA forms: oc, open circular; lin, linear; sc, supercoiled; ss single-stranded; SG ss, subgenomic ssDNA. DNA bands marked by an arrow in lane A3 are possibly dsDNA replicative forms of the SG DNA which are absent in corresponding sample in lane B2.

6.3.2 Characterisation of TLCV SG DNAs

To characterise the SG DNAs present in the D3 tomato plant (Fig. 6.1A) it was first determined whether they could be amplified with TLCV-specific oligonucleotides. A range of primers from both type and D1 strains (Chapter Three) as outlined in Table 6.2 were tested for DNA amplification and the results are shown in Fig. 6.2.

***Table 6.2** Oligonucleotide primers used to amplify TLCV and its subgenomic DNA species*

	Primers (virus)	DNA template	predicted product size	observed size
1	P1588^{c} and P1583^{v} (D1)	D3 tomato plant	Full-length, 2766 bp	Full-length, 2766 bp
2	P2639^{c}† and P272^{v} (D1)	D3 tomato plant	2368 bp	2368 bp
3	P1626^{c} and P2594^{v}‡ (type)	D3 tomato plant	1799 bp	1799 bp
4	P2386^{c} and P1^{v} (type)	D3 tomato plant	2386 bp	~ 900 and 1000 bp
5	P2639^{c} and P2640^{v} (D1)	D3 tomato plant	Full-length, 2766 bp	~ 700 bp
6	P2713^{v} and P2718^{c} (type)	agroinfected datura plant	Full-length, 2766 bp	~ 900 bp

† P2594^{v} is specific to type strain
‡ P2639 is specific to D1 strain

DNA products of expected sizes (Table 6.2, rows 1-3 and Fig. 6.2, lanes 1-3) were amplified from the D3 extract using either type or D1 strain specific primers. This indicated that the D3 tomato plant was infected with both the TLCV type and D1 strains.

With certain sets of primers (Table 6.2, rows 4 and 5 and Fig. 6.2, lanes 4 and 5) DNA products obtained from either the type or D1 strain were smaller than those predicted from the viral sequences. This was consistent with the presence of SG DNA species in the plant D3. The lack of viral DNA products in these reactions suggested that SG DNAs compete with their amplification due to their smaller size.

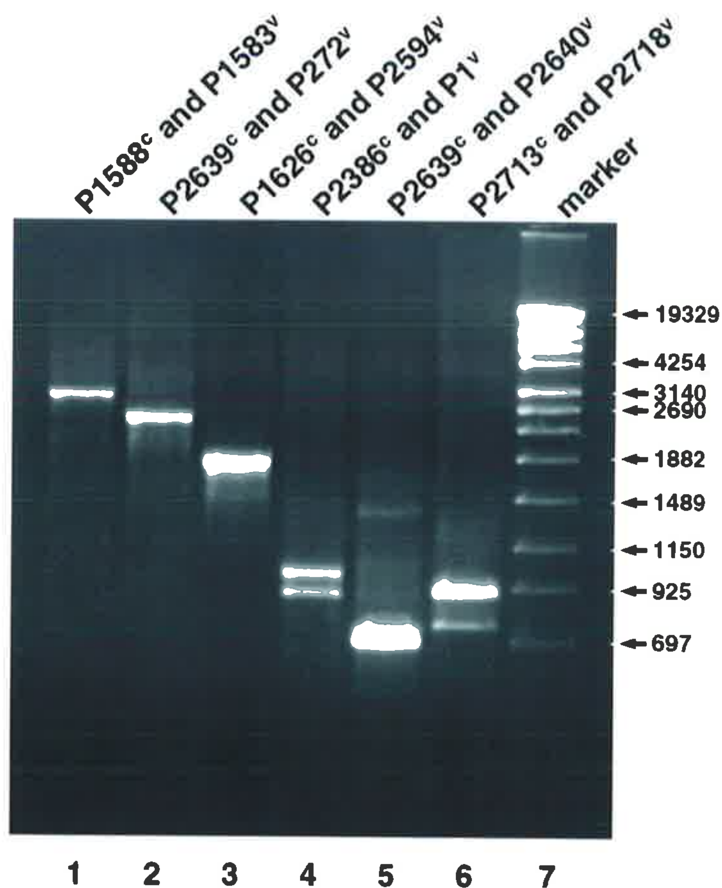


***Fig. 6.2** Amplification of TLCV strains and their subgenomic DNA species by PCR using different TLCV primer combinations.*

The absence of SG DNAs in PCR products obtained with certain primers (Fig. 6.2, lanes 1-3) indicated that one or both of these primers were located in regions of TLCV DNA which were absent in the putative SG species.

Two DNA species of approximately 900-1000 bp in size (Fig. 6.2 lane 4), appeared likely to be derived from the major SG ssDNA in the plant extract used (Fig. 6.1A, lane 1). These subgenomic DNAs were tentatively designated SG1 and SG2 according to their size, although they could not be resolved from each other by gel electrophoresis (Fig. 6.1A, lane 1). Southern blot analysis of these DNAs with a TLCV probe confirmed that they were closely related to the TLCV genome (Fig. 6.3, lane 8).

Another DNA product amplified with a different set of primers (Fig. 6.2 lane 5) was approximately 700 bp in size and appeared to be the product of amplification of the minor SG DNA present in Fig. 1A (lane 1). This SG DNA (designated SG3) is derived from the TLCV D1 strain because primer $P2639^c$ is specific to the D1 strain (Table 6.2). Regions of the TLCV genome containing the type strain primers $P2386^c$ and/or $P1^v$ (Table 6.2, row 4) must be absent in SG3 DNA, because SG3 could not be amplified with this primer combination. This conclusion was subsequently confirmed by sequencing (see section 6.3.3). In summary, these experiments showed that the D3 tomato plant contained at least three SG DNA species.

DNA amplification was also carried out on an extract of the agroinoculated datura (Fig. 6.1, lane 3). When the TLCV type strain primers $P2713^v$ and $P2718^c$ (Table 2.2) were used, which would be expected to amplify a full-length (2766 bp) TLCV product, two DNA products of approximately 700-900 bp were obtained (Table 6.2, row 6 and Fig 6.2, lane 6). These DNA species were designated SG4 and SG5. Only the larger species (SG4) was further characterised.

6.3.3 Cloning and sequencing

In order to map the SG DNA sequences on the TLCV genome, the SG1 and SG2 PCR products were subjected to restriction endonuclease digestion using enzymes with unique sites in the TLCV genome. The results (Fig. 6.3 lanes 1-7 and Table 6.3) demonstrated that all restriction sites tested, except for *Sal* I and *Sac* I, were absent in either one or both SG DNAs.

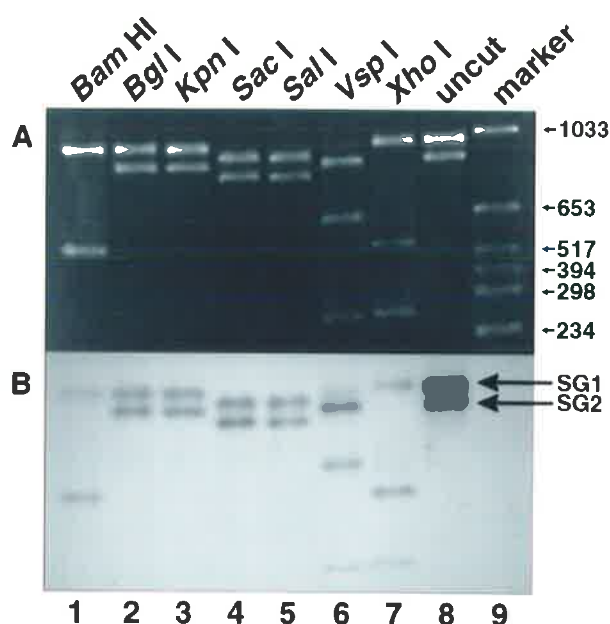


Fig. 6.3 *Restriction endonuclease analysis of PCR products of SG1 and SG2 DNAs. A) The photographic image of ethidium-stained gel containing various digestion of SG1 and SG2 DNAs obtained with primers P2386^c and P1^v (see Table 6.2, row 4). B) Southern blot hybridisation of the same gel using a ^{32}P-labelled full length probe from a TLCV clone.*

Table 6.3 *The occurrence of single restriction endonuclease sites of TLCV DNA in SG1 and SG2 DNAs.*

Restriction endonuclease	SG1	SG2
Bam HI	-	+
Bgl II	-	-
Kpn I	-	-
Sac I	+	+
Sal I	+	+
Vsp I	+	-
Xho I	-	+

Based on data outlined in Table 6.3, overlapping primers were designed to *Sac* I site to obtain full-length PCR products of SG1 and SG2 (Table 6.4, row 1). Similarly,

full-length PCR products of SG3 and SG4 DNAs were obtained using primer combinations as described in Table 6.4 (rows 3 and 5). Each PCR product was cloned and sequenced. To confirm the sequences of the first priming site of SG1, SG2 and SG4, second sets of primers were designed based on sequence data from a region carrying a unique restriction site for each SG species (Table 6.4, rows 2-4). The three DNAs were amplified, cloned and fully sequenced.

***Table 6.4** Oligonucleotide primers used to amplify and clone TLCV full-length SG DNAs.*

	Primers (virus)	*Subgenomic species*	Vector used for cloning
1	P2331^c and P2326^v (type) each containing a *Sac* I site at its 5′-end	SG1 and SG2	pBS SK$^+$ - *Sac* I cut
2	P2713^v and P2718^c (D1) each containing a *Eco* RI site at its 5′-end	SG1	pBS SK$^+$ - *Eco* RI cut
3	P2025^v and P2030^c (type) each containing a *Xho* I site at its 5′-end	SG2 and SG4	pBS SK$^+$ - *Xho* I cut
4	P2713^v and P2718^c (type) each containing a *Eco* RI site at its 5′-end	SG4	pBS SK$^+$ - *Eco* RI cut
5	P2639^c and P2640^v (D1)	SG3	pBS SK$^+$ T-tail vector

Analysis of the sequence data confirmed that the SG DNAs were derived from TLCV DNA. SG1 (1386 nt) and SG3 (699 nt) were most closely related to the D1 strain, while SG2 (1292 nt) and SG4 (903 nt) were most closely related to the type strain (Table 6.5).

***Table 6.5** Nucleotide sequence similarity between TLCV strains and their SGs in the left part of the intergenic region (151 nt).*

Subgenomic DNAs	nucleotide similarity (%)	
	TLCV type strain	TLCV D1 strain
SG1	78.8	99.3
SG2	94.0	76.8
SG3	78.8	99.3
SG4	100	80.1

A schematic drawing of the SG DNAs showing their deleted regions relative to TLCV genome is presented in Fig. 6.4. SG1 and SG2 are approximately half the size of the viral genome, SG3 is one-fourth and SG4 is one-third size of the viral genome. The number of contiguous deleted regions in each molecule varies between one to three. In SG2 DNA a 96 bp region (nucleotides 73 to 168) is repeated 3 times (Fig. 6.4B). Significantly, this extends the size of this DNA to approximately half the size of the viral genome. In SG4 DNA, a 188 bp fragment (nucleotides 1050-1270) normally located in the C2 and C3 ORF regions in the complementary-sense strand, has been inserted into the C1 ORF region in the virion-sense strand of the SG genome (Fig. 6.4D). This extends the size of this SG DNA to approximately one-third of the viral genome.

The SG DNAs contain deletions that disrupt all of the viral genes, except for the C4 ORF in SG1 and SG2 (Fig. 6.4). However, in each case the left part of the intergenic region, including Rep binding domains, are mostly conserved (see Fig 6.4) indicating that this region is essential for Rep-mediated replication of the SG molecules. Comparisons of nucleotide sequences of the left part of the intergenic region of SG DNAs with the corresponding regions of the TLCV strains showed that SG2 and SG4 contain identical sequences of the two footprints A and B including the repeat motif GGTGTCT which have been identified as binding domains for wild type Rep (see chapter 4), while SG1 and SG3 contain the repeat motif of GGAGAC which is identical to the repeat motif within the TLCV D1 *ori*.

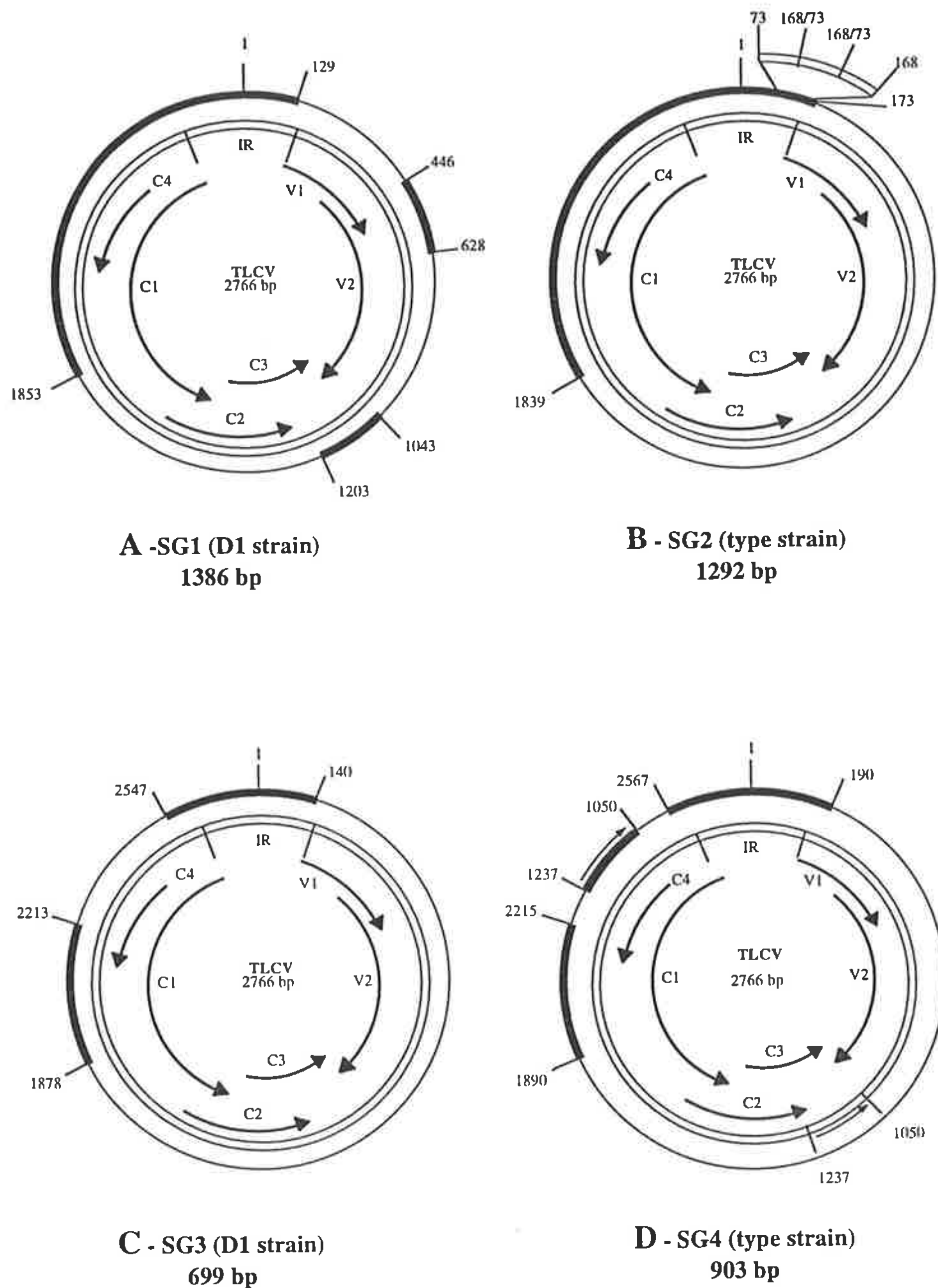


***Fig. 6.4** Schematic drawing of the TLCV SG DNAs.*

The regions of TLCV DNA present in each SG molecule are shown by the thickened sections of the outer circle surrounding the TLCV genome. Arrows display the viral ORFs and IR shows the TLCV intergenic region. In SG2, a 96 bp fragment (nucleotides 73 to 168) is repeated 3 times. In SG4, a 188 bp fragment (nucleotides 1050-1270) containing portions of C2 and C3 ORFs in the complementary-sense strand has been inserted into the C1 ORF region of the virion-sense strand. Numbering of the sequence is as in Dry et al. (1993) where residue 1 is the first nucleotide of the TAATATTAC sequence within the stem loop structure.

6.3.4 SG DNA replication in the presence of TLCV

The replication of SG2 in the absence or presence of TLCV type genome was examined using tobacco leaf strips in a transient replication assay (section 6.2.4). Southern blot analysis of DNAs extracted from leaf strips agroinoculated with a dimeric SG2 construct alone did not result in the production of any DNA form (Fig. 6.5A, lane 1). However, co-agroinoculation of leaf strips with this construct and a wild type TLCV construct led to the production of both viral and SG2 DNAs (Fig. 6.5A, lane 3). These results indicated that SG2 is absolutely dependent on TLCV for replication. Interestingly, the production of TLCV ssDNA was significantly reduced in the presence of SG2 DNA (Fig. 6.5A, compare lanes 2 and 3) suggesting that replication of SG DNA interferes with viral DNA replication. Under the experimental conditions used, TLCV did not generate SG molecules *de novo* (Fig. 6.5A, lane 2) and therefore new SG2 DNA molecules in co-agroinfected leaf strips (Fig. 6.5A, lane 3) must be generated from the inoculated SG DNA.

In the same experiment a V1/V2 deletion mutant construct of TLCV (Rigden *et al.*, 1993) was used instead of the wild type construct as the helper virus. The mutant construct produces a smaller amount of ssDNA relative to dsDNA forms compared to the wild type construct (Rigden *et al.*, 1993). When the SG2 and V1/V2 mutant constructs were agroinoculated together, SG2 could not be detected and the level of mutant ss and dsDNA forms was significantly reduced (Fig 6.5B, lane 2). These observations demonstrated that SG2 had interfered with the accumulation of mutant TLCV DNA.

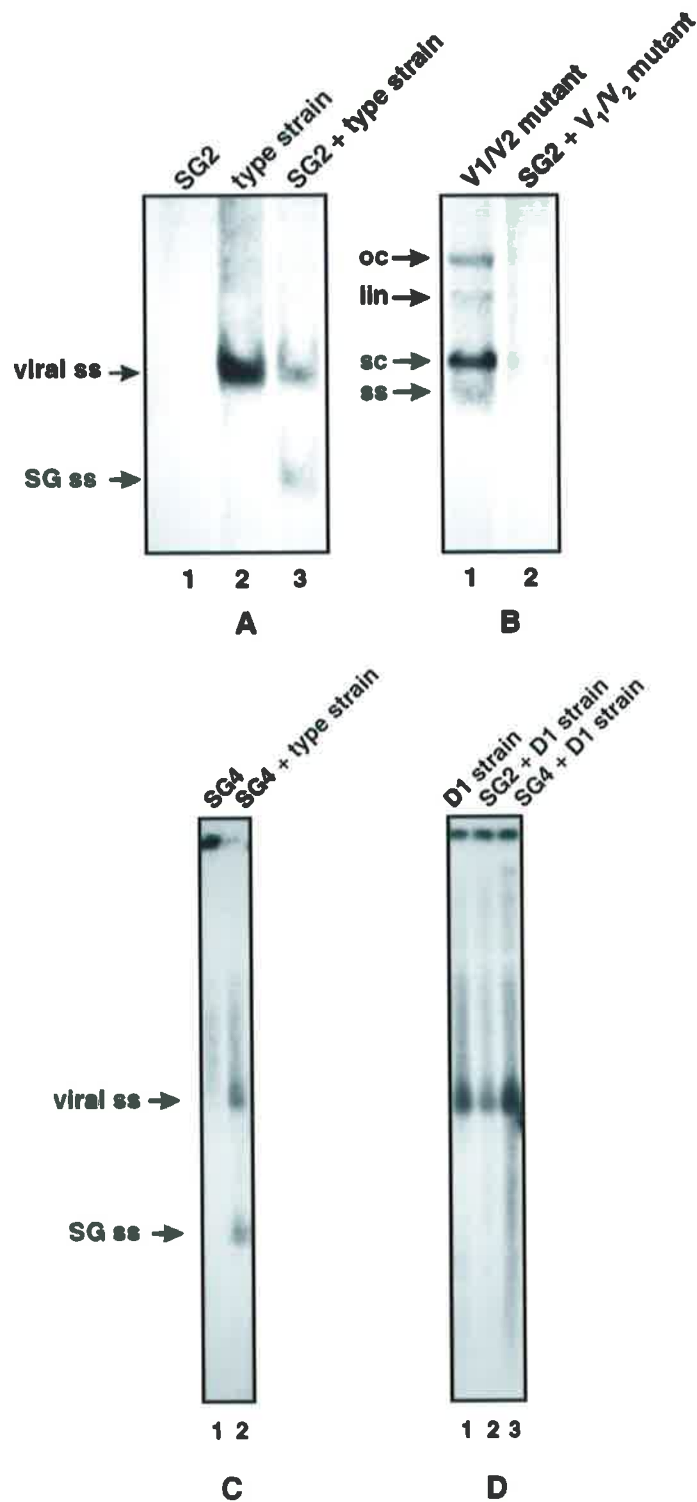


Fig. 6.5 TLCV SG DNA depends on the viral genome for replication.

Southern blot analysis of DNA (5 µg per track) extracted from tobacco leaf strips agroinoculated with SG and/or viral constructs. Various DNA forms are identified as indicated in Fig. 6.1. The TLCV V1/V2 mutant has a 510 bp deletion and therefore results in the production of smaller viral DNA forms identified on the left of track B1.

The dependence of SG replication on the helper virus genome was also examined using SG4 derived from the TLCV type strain. Mixed inoculations of a dimeric SG4 construct with the wild type TLCV construct in the leaf strip assay confirmed the same results obtained from the combination of SG2 and TLCV, showing SG4 replication only in the presence of TLCV (Fig 6.5C).

When either SG2 or SG4 derived from the TLCV type strain were co-agroinoculated with the TLCV D1 strain in the leaf strip assay, no SG DNA was observed (Fig. 6.5D) indicating that the D1 strain could not support the replication of heterologous SG2 and SG4 DNAs.

6.3.5 Are SG DNAs encapsidated?

The possibility of SG DNA encapsidation in TLCV coat protein was examined by immunocapture PCR (section 6.2.5). DNA amplification was performed using two sets of type strain primers. The first set of primers, $P272^v$ and $P1626^c$, were designed to regions of viral DNA that were deleted in SG molecules, so that only viral DNA would be amplified (Table 6.6). The second set of primers, $P1^v$ and $P2718^c$, were capable of amplifying distinctive fragments of both viral and SG DNAs (Table 6.6). The results of immuno-capture assay are shown in Fig. 6.6.

Table 6.6 *Oligonucleotide primers used to amplify the TLCV and its subgenomic DNA species in immunocapture PCR.*

Primers	Predicted viral DNA size	Predicted SG DNA size (datura)
$P1626^c$ and $P272^v$	1355 bp	-
$P2718^c$ and $P1^v$	2718	855 bp

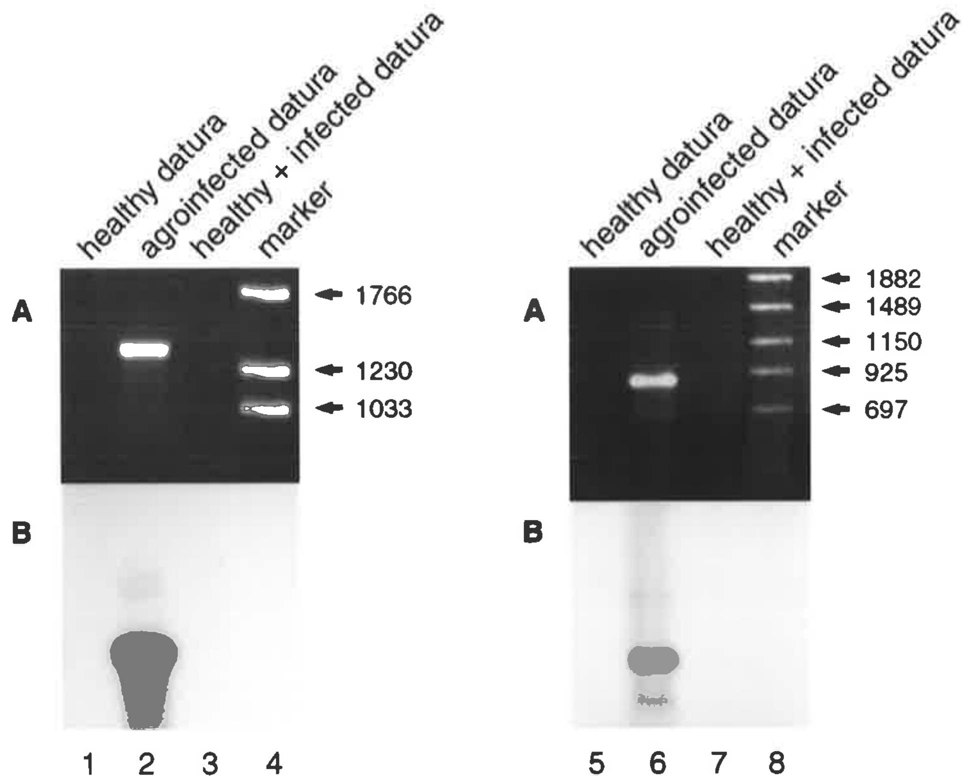


Fig. 6.6 *Encapsidation of TLCV SG DNA by the viral coat protein.*

DNA encapsidation was tested by immunocapture PCR using two sets of TLCV type strain primers: P272^{v} and P1626^{c} (left panel) prime the amplification of viral DNA only, P1^{v} and P2718^{c} (right panel) prime the amplification of partial fragments of both viral and SG DNAs. The photographic images of ethidium bromide-stained gels are shown in part A of each panel and the Southern blot hybridisation of the same gels using a ^{32}P-labelled full length TLCV probe are shown in parts B. Lanes 1, 2, 5 and 6 show PCR products obtained with template immunocaptured from extracts of healthy plants or plants agroinfected with TLCV type strain. Healthy extracts shown in lanes 3 and 7 were spiked, prior to immunocapture, with DNA extracted from infected plants corresponding to twice the weight of healthy tissue used.

When the primer combination P1626^{c} and P272^{v} (Table 6.6, row 1) was used, viral DNA products of the size expected from the viral template (1355 bp) were amplified from extracts of agroinfected datura plants (Fig. 6.6, lane 2) indicating that viral DNAs were present in particles trapped by the coat protein antibody.

With primer combination P2718^{c} and P1^{v} (Table 6.6, row 2), only an 855 bp product (Fig. 6.6 lane 6) was obtained from extracts of agroinfected datura plants. This product size is in agreement with the predicted size for amplification of the SG DNA template (Table 6.6). This amplified DNA was cloned and fully sequenced. The nucleotide sequence of this PCR product, amplified from DNA extracted from immunocaptured particles of agroinfected datura, was identical to the nucleotide sequence of SG4. These observations demonstrated that SG DNAs were present as encapsidated molecules in agroinfected datura plants. The lack of a PCR product corresponding to the viral genomic segment in Fig. 6.6, lane 6 was probably due to the competition by the smaller SG4 DNA template.

No SG PCR product was detected in the extracts of healthy datura (Fig. 6.6 lanes 1 and 5) or in the healthy extracts which had been spiked with DNA from infected plants (lanes 3 and 7). These observations demonstrated the specificity of the immunocapture step for encapsidated DNA in infected plants.

6.4 DISCUSSION

Association of smaller than unit-length DNA molecules with viral genomes is a common feature of geminivirus infections. These SG molecules often appear upon passaging virus inocula serially from infected plants to healthy plants under laboratory conditions (Czosnek *et al.*, 1989; MacDonald *et al.,* 1988; MacDowell et al., 1986; Stanley and Townsend, 1985). In the case of BCTV, it has been reported that SG DNA

molecules are produced from cloned inocula on the first passage (Frischmuth and Stanley, 1992; Stenger *et al.*, 1992). Like BCTV, TLCV produces SG DNA molecules consistently from cloned inocula in the first passage. TLCV SG DNAs accumulated in agroinfected datura and *N. benthamiana* tissues within 5 to 12 weeks after inoculation, whereas agroinfected tomato plants did not support the production of SG DNAs even one year after inoculation. These observations indicate that production of SG DNAs *de novo* by cloned TLCV DNA inocula is influenced by the host plant.

Interestingly, three of the TLCV SG DNAs reported here (SG1, SG2 and SG3) were isolated from a tomato plant grafted with field-infected material. Since TLCV SG DNAs were not produced in agroinoculated tomato plants (Fig. 6.1A, lane 2), it is possible that the SG DNAs observed in the naturally infected tomato plants originated from another host and were transferred to tomato through inoculation by the whitefly vector. Naturally occurring SG DNAs in field-infected material have also been reported to be associated with BCTV (Stenger, 1995) and ACMV (Coutts and Buck, 1987).

The mechanism by which SG DNAs are generated in geminiviral infected plants is unknown. SG DNAs seem to be produced as a result of large sequences deletions during DNA replication. It has been suggested that such deletions may be introduced by polymerase jumping, copy choice mechanisms or illegitimate recombination events (Frischmuth and Stanley, 1992; Stenger *et al.*, 1992). SG DNAs may contain single or multiple (2-3) deletions (see Fig. 6.4). In some geminiviruses, the deletions are flanked at the junction by short repeat sequences of 2- to 7-bp in length (Frischmuth and Stanley, 1992; Stanley and Townsend, 1985; Stenger *et al.*, 1992). However, such repeat sequences were not present at the boundaries of deletions in TLCV SG DNAs.

Apart from BCTV, a subgroup II geminivirus which produces a heterogeneous

population of SG DNAs ranging in size from 0.8 to 1.8 kb (Frischmuth and Stanley, 1992; Stenger *et al.*, 1992), other geminiviruses normally generate SG DNAs that are approximately half-size of the respective viral genome (MacDonald *et al.,* 1988; MacDowell et al., 1986; Roberts *et al.,* 1988; Stanley and Townsend, 1985). The results of this study showed that TLCV produced multiple SG DNAs of different sizes independently in a single plant. The size of TLCV SG DNAs were approximately 1/2, 1/3 or 1/4 of the size of the full-length viral genome. This may indicate a size selection by encapsidation where multiple SG molecules are encapsidated within a single TLCV capsid.

The possible dependence of TLCV SG DNA encapsidation on molecular size is reflected not only by the presence of DNA molecules of different size rather than heterogeneous populations, but also by DNA rearrangements involving either DNA insertions (Fig. 6.4D) or repeats (Fig. 6.4B) resulting in SG DNA sizes which are presumably necessary for encapsidation. It has been reported (Dry *et al.*, 1997) that a circular ssDNA satellite associated with TLCV infection is encapsidated by TLCV coat protein and spread in infected plants. The size of this DNA is 682 nt, approximately 1/4 of the size of the TLCV genome, possibly the result of size selection for encapsidation (Dry *et al.*, 1997).

Southern blot analysis of DNA extracts of a tomato plant naturally infected with TLCV indicated that SG DNAs were composed of predominantly ssDNA occurring at the same or higher levels compared to viral ssDNA and probably constituting a large proportion of the virions produced. Evidence obtained by immuno-capture PCR supports the view that SG DNAs are encapsidated by TLCV coat protein. This suggestion is in agreement with the observation of SG DNAs associated with purified

ACMV particles, indicating that SG DNAs are encapsidated within isometric particles (Stanley and Townsend, 1985).

Geminiviral SG DNAs are invariably defective and rely upon the wild-type genome for replication. In all four TLCV SG DNAs characterised in this study, all essential viral ORFs for replication and encapsidation were disrupted (Fig. 6.4). The SG DNAs were not therefore expected to be capable of autonomous replication, and this was confirmed by an *in vitro* replication assay (Fig. 6.5). However, they were replicated in the presence of the viral DNA (Fig. 6.5A, lane 3) and did not appear to accumulate merely as an end-product of TLCV DNA replication.

In all of the SG DNAs characterised, the intergenic region including the viral origin of replication was present. This suggests that the intergenic region, which includes the Rep binding domains, is essential for replication. It should also be noted that each SG DNA was replicated in the presence of the respective helper virus strain, but not by a heterologous strain. This indicates that SG replication, like that of the viral DNA, involves the highly specific interaction of the Rep-binding elements with the cognate viral Rep (see Chapter 4). Choi and Stenger (1995) have previously shown that a transgenic BCTV-derived SG DNA could only be mobilised and replicated in transient replication assays in the presence of the helper virus strain from which the SG DNA was derived.

SG DNAs have been shown to interfere with viral replication and infectivity (Frischmuth and Stanley, 1991; MacDowell et al., 1986; Stanley and Townsend, 1985). In this study evidence of an interfering effect of SG DNAs on viral DNA replication was obtained in the leaf strip transient replication assay (Fig. 6.5). Stanley *et al.* (1990) have shown that ACMV-induced disease symptoms are attenuated in plants transformed with a

tandem repeat of an ACMV-derived SG DNA. They suggested that in these transgenic plants, unit-length SG DNA was released from the chromosome and amplified in the presence of the wild-type virus. Similarly, Frischmuth *et al.* (1997) recently showed that *N. benthamiana* plants transformed with a partial repeat of a SG DNA derived from BCTV produced ameliorated symptoms when agroinoculated with BCTV. On the basis of these observations, the geminivirus SG DNAs have been recognised as defective-interfering DNAs.

The role of SG DNAs in the geminivirus mutiplication cycle is unknown, but they may act to reduce virus replication levels in host plants and hence enhance the survival of the virus-infected host. Infected plants with reduced disease symptoms may favour acquisition of the virus by insect vectors and increase the chances that the virus spreads (Bisaro, 1994).

Chapter Seven

Summary and Conclusions

The family *Geminiviridae* (Briddon and Markham, 1995) comprises unique genera of plant viruses characterised by small twinned isometric particles containing either one or two circular ssDNA species. Their genomes are thought to replicate by a rolling circle replication mechanism. They contain a small number of genes arranged in two different clusters separated by an intergenic region which includes a sequence capable of forming a stable hairpin loop structure containing the motif 5′-TAATATTAC-3′, which is common to all geminiviruses. Geminiviruses encode a replication associated protein (Rep), which is absolutely required for replication of viral DNA (Laufs *et al.*, 1995). The viral origin of replication (*ori*) has been mapped to the left side of the intergenic region (Laufs *et al.*, 1995).

Geminiviridae have been classified into three *genera* based on host range, type of insect vector and genome organisation (Briddon and Markham, 1995). Subgroup I is comprised of leafhopper-transmitted viruses that infect monocotyledonous hosts and have monopartite genomes. Subgroup II includes viruses that, like subgroup I viruses, are transmitted by leafhoppers and have a monopartite genome, but infect dicotyledonous hosts. Subgroup III is the largest subgroup and contains viruses that are transmitted by a single species of whitefly, *B. tabaci*. They have either a bipartite or monopartite genome and infect dicotyledonous hosts. The subgroup III viruses form two distinct clusters according to their geographical origins in either New World or Old World countries (Padidam *et al.,* 1995).

Tomato leaf curl virus, a monopartite subgroup III geminivirus, causes a serious disease of tomato in the northern parts of Australia. Infected tomato plants show varying degrees of stunting depending on how early they were infected, as well as leaf curling, yellowing, upward leaf rolling and cessation of fruit production.

The major aims of the work reported in this thesis were: (1) to characterise new strains of tomato leaf curl virus; (2) to study the interaction between TLCV Rep and the viral DNA; (3) to determine genomic DNA elements defining strain specificity and (4) to characterise TLCV subgenomic DNAs.

Three distinct virus-like agents which were isolated from two tomato plants graft-inoculated with a symptomless wild *Solanum* species are described in Chapter Three. Two of these agents were found to be new strains of TLCV, designated the D1 and D2 strains. This wild *Solanum* sp. may be an overwintering virus reservoir in Northern Australia. The third infectious agent was found to be a previously undescribed strain of potato spindle tuber viroid. This is the first report of a virus-viroid co-infection involving a geminivirus, a phenomenon with potential biological implications for the replication and spread of these two agents within the same host plant. However, there appeared to be no inhibitory interaction between replication of TLCV and PSTVd in co-inoculated plants. Previous reports of PSTVd-virus interaction have only involved RNA viruses, e.g., potato leaf roll virus (Salazar *et al.*, 1995).

The D1 and D2 strains of TLCV had an overall nucleotide sequence similarity of approximately 95% with the TLCV type strain, but their sequence similarity with the TLCV type strain within the intergenic region was only 86%. It has been proposed that geminiviruses having greater than 90% nucleotide sequence similarity be considered as strains (Czosnek and Laterrot, 1997; Padidam *et al.,* 1995). On this basis, the D1 and D2

viruses are distinct strains of TLCV. The greatest degree of similarity between the new TLCV and type strains was observed in the V2 (coat protein) ORF, whereas the highest degree of divergence was observed in the C1 ORF (Rep).

The work described in Chapter Four provides the first direct evidence of geminiviral Rep:DNA binding, an important step in the replication cycle of this group of plant viruses. TLCV Rep was expressed in *E. coli* as a histidine-tagged fusion protein, purified to apparent homogeneity in soluble form and used in various *in vitro* binding experiments with different fragments of the TLCV genome. Electrophoretic mobility shift assays demonstrated that the Rep binds specifically to a 120 bp fragment within the left part of the TLCV intergenic region.

DNase I footprint analysis of the binding regions within the 120 bp fragment demonstrated two closely positioned footprints (A and B) covering the sequences GCAATTGGTGTCTCTCAA and TGAATCGGTGTCTGGGG, which contain the direct repeat of the motif GGTGTC (underlined). The footprints are located between the start codon of the C1 ORF and the C1 promoter TATA box (Fig 7.1). The results suggest that the repeat motif is involved in virus-specific Rep binding, but may not constitute the entire binding element.

Fig. 7.1 *Location of the Rep binding elements (A + B) within the left hand side of the TLCV intergenic region.*

As discussed in Chapter Five, mixed agroinfection experiments conducted in whole plants using D1 and type strains indicated that both viruses can replicate simultaneously within the same host plant. However, the D1 strain could not complement a replication-defective mutant of the type strain under conditions where complementation of the mutant could be demonstrated with another mutant of the type strain. This indicates that each strain is replicated independently in co-infected plants by its own specific Rep. Using electrophoretic mobility shift assays, it was further demonstrated that the Rep of the type strain does not bind to the *ori* of the D1 strain. DNase I footprinting technique experiments confirmed that the Rep-binding elements within the TLCV *ori* are strain-specific.

In addition to the full-length DNA forms of the TLCV genome, subgenomic DNA molecules of varying size were found in plants naturally infected with TLCV (Chapter Six). Furthermore, agroinfection of plants with cloned TLCV DNA resulted in the *de novo* production of subgenomic DNA forms in the inoculated plants. Two discrete subgenomic DNAs, derived from each of the TLCV type and D1 strains, were fully sequenced and found to contain deletions which disrupt all the viral genes required for replication, coat protein synthesis and viral spread. However, all subgenomic DNA forms contained the viral *ori* including the Rep binding domains essential for replication. It was further shown that subgenomic DNAs are encapsidated by TLCV coat protein using an immunocapture PCR technique. Co-agroinoculation of tobacco leaf strips with *A. tumefaciens* containing tandem repeat constructs of viral and subgenomic DNAs indicated that subgenomic DNAs are strictly dependent for replication on the homologous virus strain, and that they are not end-products of TLCV replication. These

results also showed that subgenomic DNAs interfered with the replication of the viral genome and, as such, may be classed as defective-interfering DNAs.

The replication of TLCV subgenomic DNA only by its homologous helper virus is consistent with the specificity of interaction of the Rep with its cognate *ori* as demonstrated by direct binding experiments. Rep recognition of TLCV DNA may be regarded as a key point for sequence divergence and viral speciation, and cross interaction between Rep protein and viral DNAs may be considered as a criterion for defining virus species.

APPENDIX A

Sequence alignment of the full-length DNAs of the TLCV strains. Dots indicate that the residues are the same as the nucleotides in the top sequence.

<u>Strains</u>

```
Type    TAATATTACCGGATGGCCGCGAAAAAATAAAGTGGTCCCCCCCACGTGATTGATGT-GAC 60
D1      ........................................T.-......A......C... 60
D2      ..........................................-.............T... 60

Type    CTGTCGACGAATGAGAACCGCGCGTCATCGCTTATTTAAGTTTTTTGTCGTATATATACT 120
D1      TG...............T...............................T.......... 120
D2      TG.....................C..........GA...T.A...............G.. 120

Type    TGGGCCCTAAGTATTTAGGCCCATAAAATGTGGGATCCTTTAGTCCACGAATTTCCTGAA 180
D1      ....................TT.-.................................... 179
D2      .........................................................A.. 180

Type    ACCGTTCACGGTTTCCGTTGTATGCTCGCCAACAAATATTTGTTGGCTGTTGAATCAAAA 240
D1      .........................................A......A........... 239
D2      ............................................................ 240

Type    TACGCTCCGGATACATTGGGATACGAATTGATCAGGGATTGCATTGGGGTTGTTAGGTCT 300
D1      ....................C....................................... 299
D2      ....................T....................................... 300

Type    CGAAATTATGAGCAAGCGACCAGCAGATATCGTGATATCTACACCCGCCTCCAAGGTGCG 360
D1      ............................................................ 359
D2      ............................................................ 360

Type    ACGGAAGCTGAACTTCAACAGTCCGTTCAAGAGCGCTGCTGCTGTCCCCACTGTCCGCGT 420
D1      ............................................................ 419
D2      ............................................................ 420

Type    CACAAGAAGGCGGACATGGGTGAATCGGCCCATGTACAGAAAGCCCATGATGTACAGGCT 480
D1      .....................................C...................... 479
D2      ............................................................ 480

Type    GTTCAGAAGCCCTGATGTTCCACGGGGCTGTGAAGGCCCATGTAAGGTCCAATCATATGA 540
D1      ...................................................G........ 539
D2      ................................................T........... 540

Type    GCAGCGTCATGATGTGGCCCATGTGGGTAAGGTCCTTTGTGTTTCTGATGTCACTAGGGG 600
D1      .......................................................C.A.. 599
D2      A.......................C.............................A..... 600

Type    TACTGGAATAACGCATCGCACTGGGAAGAGATTTTGTATTAAATCTATTTATGTCCTGGG 660
D1      ............T.........................................TT.... 659
D2      ...G........T...................................A.....T..... 660

Type    CAAGATCTGGATGGATGATAACATTAAGACTAGGAACCATACGAATACAGTTATGTTCTT 720
D1      T.................C...........G............................. 719
D2      ......A...........C.....C...........T....................... 720
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Type  CTTGGTCCGTGATAGGAGGCCCTATGGGACTCCTAAGGATTTTGGGCAGGTGTTCAACAT 780
D1    .................................G....................T..T.. 779
D2    ..........................................G........C........ 780

Type  GTATGACAATGAGCCCAGCACAGCTACTGTGAAGAACGACATGAGGGATGGCTTTCAAGT 840
D1    ..............................T..........................G.. 839
D2    ..................T..T.......C......T..T.................... 840

Type  TATCAAGAAGTGGTCCGCAACTGTCACTGGTGGGCAATATGCGTCTAAGGAGCAGGCTAT 900
D1    ............................................................ 899
D2    .............................................C...........G.. 900

Type  TATCAATAGGTTTTATAAAATATATAACCATTGTACTTACAATCATCAGGAAGCTGCAAA 960
D1    ............................................................ 959
D2    ...T.....A...........T.....T...........T.................... 960

Type  GTATGAGAATCATACTGAGAATGCATTATTATTGTATATGGCATGTACACATGCCTCTAA 1020
D1    ...............G................................C........... 1019
D2    ............................................................ 1020

Type  CCCAGTGTATGCTACGTTGAAGATACGGATCTATTTCTATGATTCGATTCAGAATTAATA 1080
D1    ...............................................CC........... 1079
D2    ...............T.....A...................................... 1080

Type  AAAATTAAATTTTATATCATGATCTTCAATTACATCAAATGTTGAGTCTAGTACATTATA 1140
D1    .......................T...G.................C....A.....C... 1139
D2    .......T.............G.T...G.................C..C.A......... 1140

Type  CAATACATGATCAACTGATCTAATTACATTGTTAATGCTAATAACTCCTAAACTATCTAA 1200
D1    ..................C................................C........ 1119
D2    ............................................................ 1200

Type  ATACTTCATGACTTGGGTCTTAAATACCCTCAAGAAACGCCAAGTCGCTGGTTGTAAGCG 1260
D1    ............................................................ 1259
D2    ...........T................................................ 1260

Type  AGTGAAAACTGTGAAGTCCAGGAAGCATTTGTGTATCGCCAATGCTCTCCTCAGGTTGTG 1320
D1    ....................T................C...................... 1319
D2    .........CT....A.....................C........T............. 1320

Type  ATTGAACTGTATCCGGACTTGAATGATGTCGTGGTTGTAGTTGAACGGGCGCTTGTGGTG 1380
D1    .....................................................G...... 1379
D2    .....................................................G...... 1380

Type  CTTTATGATCTTGAAATAGAGGGGATTTGGAACCCTCCAGATATACTCGCCATTCATCGC 1440
D1    ..C..........................................T..T........... 1439
D2    ............................................................ 1440

Type  TTGACGAGCAGTGATGGGTTCCCCTGTGCGTGAATCCATGGTTATGGCAGTTGATATGCA 1500
D1    ......................................................A..... 1499
D2    ...........................................G..........A..... 1500

Type  GGTAGTAAGAACACCCGCACGGCAAATCAATTCTCTTCCTCCGGATGGACCTCTTCTTCG 1560
D1    .....................T...G.................................. 1559
D2    .........................G.................................. 1560
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Type  CTATTTTGTGTTGGACCTTGATAGGTACCTGAGTATAGTGGCTCGTTGAGGGTGATGAAT 1620
D1    ...................................C.AC..................... 1619
D2    ...................................C.AC.......C..A.......... 1620

Type  TCTGCATTTTTTAAAGCCCAGGCTTTTAATGCGCTGTTTTTCTCCTCATCGAGGTACTCT 1680
D1    ............................................................ 1679
D2    ......................................C..T.................. 1680

Type  TTATAGCTGGAGTTGGGTCCTGGATTGCAGAGGAAGATAGTGGGTATTCCACCTTTAATT 1740
D1    ......................................T..................... 1739
D2    ............................................A............... 1740

Type  TGAACTGGCTTGCCGTACTTGGTGTTGCTTTGCCAATCTCTCTGGGCCCCCATGAATTCT 1800
D1    .................T.......................................... 1799
D2    ........T..........................G....G................... 1800

Type  TTGAAATGTTTCAGATAATGCGGATCAACGTCATCAATGACGTTATACCACGCATCATTT 1860
D1    ............................................................ 1859
D2    .............A.........G..C.......................T......... 1860

Type  GAATACACTTTAGGGCTTAAATCAAGGTGCCCACATAAATAATTATGTGGACCCAATGAA 1920
D1    ..G......................................................... 1919
D2    .....T...........C.............................G..G........C 1920

Type  CGCGCCCAAACAGTCTTCCCTGTTCTAGACTCACCCTCTATTACTATACTTATGGGTCTC 1980
D1    .......................C.................................... 1979
D2    .......................C.................................... 1980

Type  AAAGGCCGCGCAGCGGCATCCTTCACATTCTCGGCGACCCACTCCTCGAGTTCTTCTGGA 2040
D1    ..T............................................C...A........ 2039
D2    ..C.............G..............................C...A........ 2040

Type  ACTCGATCAAAAGAAGAAGATAAAAAAGGAGAAACATAAACCTCCAACGGAGGTGTAAAA 2100
D1    .....................G..........................A........... 2099
D2    .....................G...................................... 2100

Type  ATCCTATCTAAATTACTATTTAAATTATGAAATTGTAAAACATAATCCTTAGGGGCTAAT 2160
D1    .............................G.............................. 2159
D2    ............................................................ 2160

Type  TCCCTAAGGACGTTAAGAGCCTCTGACTTACTTCCAGTGTTAAGCGCCTGGGCGTAAGCG 2220
D1    ............................................................ 2219
D2    ............................................................ 2220

Type  TCATTGGCTGATTGTTGTCCCCCTCTTGCAGATCGTCCATCGATCTGAAACTCTCCCCAT 2280
D1    ............................................................ 2279
D2    ............................................................ 2280

Type  TCGAGGGTGTCTCCGTCCTTCTCCAGATAGGACTTGACGTCTGACGAGCTCTTAGCTCCC 2340
D1    ............................................G....C.......... 2339
D2    ............................................................ 2340

Type  TGAATGTTCGGATGGAAATGTGCTGACCTGGTGGGGAGACCAGGTCGAAGAATCGTTGA 2400
D1    ......G.................................................... 2399
D2    ........................................................... 2400
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Type    TTCTTGCACTGGAACTTGCCTTCGAACTGGATAAGCACGTGGAGATGAGGCTCCCCATTC 2460
D1      ...G....GAC.T.T............................................. 2459
D2      ...G....GAC.T.T............................................. 2460

Type    TCATGGAGTTCTCTGCAAACTTTAATATATTTTTTGGATGTTGGGGTTTCTAGGTTATTT 2520
D1      ...................T...G.GG........ATTA........A.T..AAGAC.GA 2519
D2      .................G.T...G.GG........ATTA........A.T..AAGAC.GA 2520

Type    AATTGGGAAAGGGCTTCTTCTTTAGTGAGAGAGCATTTGGGATAAGTGAGGAAATAATTC 2580
D1      .G.........T........C..T.A.........C..A....T....A..GA...T 2579
D2      .G.........T........C..T.A.........C..A....T....A..GA...T 2580

Type    TTAGCATTTATACGGAATGACTTTGGTCTAGTCATTGC-GTTTTAGCAATTGGTGTCTCTC 2640
D1      ..G..T.G...T.TA..ACG...A..GGGT.C...GT.A.CAAA.TG......A.A.AA.. 2640
D2      ..G..T.G...T.TA..ACG...A..GGGT.C...GT.A.CAAA.TG......A.A.AA.. 2641

Type    AA-CTTGGTAAATGAATCGGTGTCTGGGGTCTTATTTATAGTTGGACACCGTATGGCATTT 2700
D1      ..A....C.........T..A.A....A..ACA..A.....G..-T.T..AA......... 2700
D2      ..A....C.........T..A.A.A..A..ACA..A.....G..-T.T..AA......... 2701

Type    TGGTAATTCGGAGAATTCTCAAAAGTAAATAATTCAAAATTCAAATCCCTCAAGCGGCCA 2760
D1      ........T....................................AT............. 2760
D2      ........T....................................AT............. 2761

Type    TCCGTC 2766
D1      ...... 2766
D2      ...... 2767
```

APPENDIX B

Sequence alignment of the full-length RNAs of the PSTVd-D sequence variants. Dots indicate that the residues are the same as the nucleotides in the top sequence.

```
Sequence
Variants

PSTVD-D1 - CGGAACUAAACUCGUGGUUCCUGUGGUUCACACCUGACCUCCUACCCAGA -50
PSTVD-D2 - ...........................A......................

PSTVD-D1 - AAAGAAAAAAGAAGGCGCUCGGAGGAGCGCUUCAGGGAUCCCCGGGGAAA -100
PSTVD-D2 - ..................................................

PSTVD-D1 - CCUGGAGCGAACUGGCAAAAGGCGCGGUGGGGAGUGCCUCGCGGCCGACA -150
PSTVD-D2 - ..................................................

PSTVD-D1 - GGAGUAAUUCCUGCUGAAACAGGGUUUUCACCCUUCCUUUCUUCAGGUUU -200
PSTVD-D2 - ..................................................

PSTVD-D1 - CCUUCCUCGCGCCCGCAGGAUCACCCCUCGCCCCCUUGCGCUGUCGCUUC -250
PSTVD-D2 - ..................................................

PSTVD-D1 - GACUACUACCCGGUGGAAACAACUGAAGCUCCCGAGAACCGCUUUUUCUC -300
PSTVD-D2 - .G................................................

PSTVD-D1 - UAUCUUGCUGGUGUCGGGGCGAGGGUGUUUAGCCCUUGGAACCGCAGUUG -350
PSTVD-D2 - .............................................U....

PSTVD-D1 - GUUCCU -356
PSTVD-D2 - ......
```

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