Characterisation of transgenic plants transformed with the *c4* gene from tomato leaf curl virus

by

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Summary

The genome of the tomato leaf curl virus from Australia (ToLCV), like other geminiviruses, has functional genes on both the virion-sense and complementary-sense strands of the replicative form of its DNA. The complementary-sense strand contains four overlapping genes (*c1-c4).* The *c1* gene is essential for viral DNA replication and its sequence contains the entire *c4* gene within a different open reading frame. The aim of the research was to express the isolated *c4* gene in transgenic plants to determine its function *in planta* and examine its potential use in a virus resistance strategy.

Leaf and stem explants from *Nicotiana tabacum* variety Samsun and *Lycopersicon esculentum* var. Ailsa Craig were used to produce transgenic plants following *Agrobacterium-* mediated transformation with a gene construct containing the *c4* gene from ToLCV under the control of a 35*S Cauliflower mosaic virus* promoter. The regenerated transgenic plants that expressed high levels of the *c4* transgene developed into abnormal phenotypes with virus-like symptoms including deformed leaves with puckering, blistering, twisting and curling. Some plants also displayed zig-zag growth characterised by change in direction at alternative nodes, thickened stems, enations and tumorous growths on deformed flowers. In contrast, similarly prepared transgenic tobacco and tomato plants with a frame-shift version of the *c4* gene construct (*c4 f-s*) grew normally in all respects, despite the fact that these plants produced a similar level of transgene RNA as the abnormal plants expressing the native *c4* gene. These results indicated that the abnormal transgenic phenotypes resulted directly from the expression of the C4 translation product, rather than the expression level of *c4* transgene *per se.* These findings substantiated a previous hypothesis that the C4 open reading frame of ToLCV encodes a protein involved in a process leading to the development of disease symptoms during viral infection (Rigden *et al.,* 1994).

The regenerated kanamycin-selected plants of the T_1 generation were tested for resistance to ToLCV via the *Agrobacterium*-mediated infection method of Grimsley *et al.,* 1986. None of the transformed lines were immune to infection by ToLCV-DNA at inoculum levels of 0.56 x 10^{-3} OD_{600} units and higher. However, several lines of the transformed tobacco and tomato with *c4* and *c4 f-s* gene constructions showed the ability to resist or tolerate infection by ToLCV-DNA via agro-infection at a diluted level of inoculum (0.56 x 10^{-3} OD_{600} units) that induced a 100% infection rate in the similarly treated control Samsun and Ailsa Craig plants. The resistance and tolerance characteristics displayed by some of the transformed lines included the absence of virus infection or absence of symptoms, delayed development of disease symptoms and lower levels of viral DNA replication relative to the untransformed plants. Several transformed lines appeared to be worthy of further testing by whitefly-mediated viral infection.

Declaration

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

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Publications

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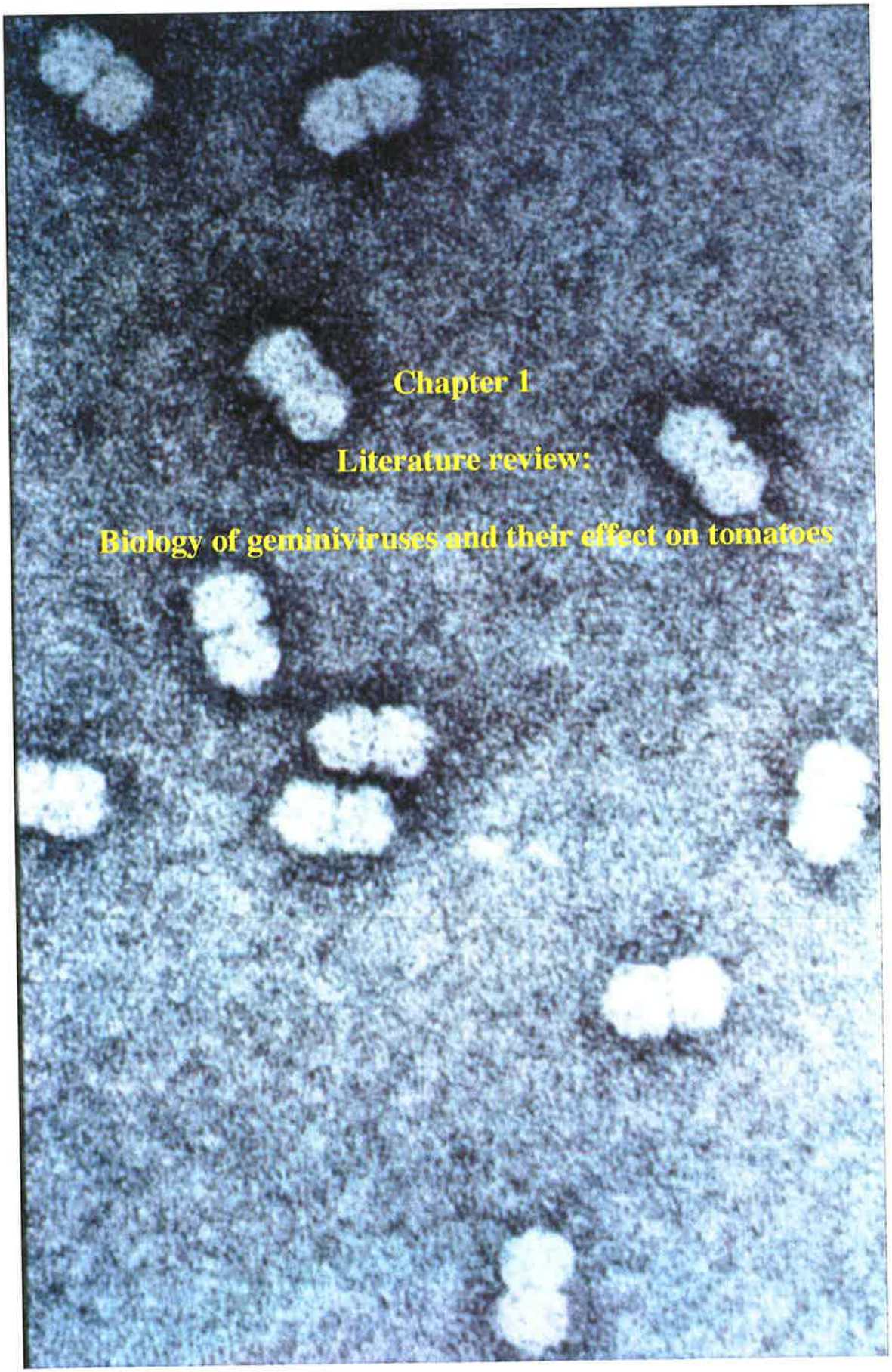
Abbreviations

A	adenine
ATP	Adenosine-triphosphate
BA	6-benzylaminopurine
bp	Base pair
BSA	Bovine serum albumin
C	cytosine
CIP	Calf intestinal alkaline phosphatase
dATP	2'-deoxyadenosine-5'-triphosphate
dCTP	2'-deoxycytosine-5'-triphosphate
dGTP	2'-deoxyguanosine-5'-triphosphate
dTTP	2'-deoxy-thymidine-5'-triphosphate
dNTP	Equimolar mixture of 2'-deoxyribonucleoside-5'-triphosphates
dd-	dideoxy-
DF	degrees of freedom
ds	double-stranded
EDTA	ethylenediamine tetra acetic acid
F	F ratio (mean square divided by the error mean square)
G	guanine
g	gram
g	unit of acceleration equal to gravity
IAA	indole-3-acetic acid
IPTG	iso-propyl-β-D-thiogalactopyranoside
Kb	kilobases
kDa	kilo-Daltons
LB	Luria Bertani medium
M	Molar
mA	milli-ampere
mg	milligram
ml	millilitre
mm	millimetre
Mr	Molecular weight
MS	Mean sums of squares
N	Normal concentration
NAA	α-naphthaleneacetic acid
ng	nanogram
nm	nanometre

Abbreviations (continued)

nt	nucleotide
ORF	open reading frame
P	Probability
PCR	Polymerase chain reaction
p-i	post-inoculation
rpm	revolutions per minute
SDS	Sodium dodecyl sulphate
ss	single-stranded
SS	Sums of squares
T	thymine
TEMED	*N, N, N'-N'*-Tetramethylethylenediamine
TRIS	Tris(hydroxymethyl)methylamine
U	unit
µCi	micro-Curie
µl	microlitre
µf	micro-faraday 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

Abbreviations for genes and gene products used throughout this text follow the guidelines suggested by the ISPMB Commission on Plant Gene Nomenclature (Price *et al.*, 1996). Also, the conventions currently used by the International Committee on Taxonomy of Viruses (ICTV) for naming the open reading frames of sequenced viruses have been adopted (Van Regenmortel *et al.*, 2000).



Chapter 1

Literature review:

Biology of geminiviruses and their effect on tomatoes

1.1 Introduction

Geminiviruses damage crops in tropical and subtropical regions through the activity of viruliferous vector insects including leafhoppers (*Cicadellidae*), treehoppers (*Micrutalis malleifera*) and whiteflies (*Bemisia* species) (Storey, 1924; Bird and Maramorosch, 1978; Bock, 1982; Briddon *et al.*, 1996). In recent decades, the range of plant species threatened by geminiviruses has widened to the extent that crops including maize, cassava, sugarbeet, sugarcane, wheat, tobacco, cotton, okra, bean, cowpea, potato, pepper, squash, watermelon and tomato are now all at risk. The trade in plants between distant geographical regions has increased the risk from introduced plant viruses and the insects that transmit them. These pests and pathogens flourish in a monoculture of susceptible crops. A new outbreak of a geminivirus in any agro-ecosystem is influenced by environmental factors, the presence of alternative plant host species that may become bridging reservoirs of virus from one crop to the next and agricultural practices that affect the multiplication of insect vectors. Chemical control of insects that transmit geminiviruses is becoming less effective in recent years with the emergence of biotypes with greater and broader resistance to insecticides. New methods for control are now needed and there is widespread interest in the development of plant varieties resistant to viruses. Genetically engineered pathogen-derived resistance is being utilised to complement classical breeding programs. The refinement of these procedures requires a systematic analysis of the function of viral genes and an understanding of the interactions between these genes and the host plant. This knowledge may lead to the development of new control strategies.

Some progress has been made in resolving the mechanisms involved in plant infection by geminiviruses. These viruses cannot infect a plant until they are able to cross the

barrier of the plant cell wall. Once inside a susceptible cell, these viruses appear to utilise host factors to mediate their replication. Geminiviruses vary in their ability to invade different plant tissues and some species appear to be restricted to the phloem (Cherif and Russo, 1983; D'Hondt and Russo, 1985). At the cellular level, cytopathological abnormalities in the nucleus of infected cells may precede the accumulation of viral particles and may reflect synthesis of viral transcripts (Kim *et al.*, 1978). These changes include the repositioning of chromatin to the periphery of the nuclear membrane and the presence of hypertrophic nucleoli appearing as granular and fibrillar structures (Adejare and Coutts, 1982; Francki *et al.*, 1985; Credi *et al.*, 1989; Gallitelli *et al.*, 1991). The virions appear to accumulate later and form irregular aggregates or crystalline arrays in the nuclei, or cytoplasm, or chloroplasts, or in the vacuoles of infected cells (Francki *et al.*, 1985; Davies and Stanley, 1989; Brunt *et al.*, 1990).

1.2 Nomenclature and taxonomy of *Geminiviridae*

The name geminivirus (Harrison *et al.*, 1977) was adopted to describe viruses having the appearance of twinned quasi-isometric particles (illustrated in Figure 1). Each geminate particle is approximately 18 x 30 nm in size, consisting of 110 protein subunits of a structural coat protein (Mr 28-34 X 10^3) surrounding one molecule of virion-sense single-stranded DNA ranging in size between 2.56~3.08 kilobases (Tables 1 to 4).

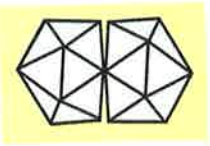


Figure 1. The shape of a geminivirus particle is similar to a pair of fused pentameric icosahedra.

The International Committee on Taxonomy of viruses (ICTV) has established guidelines for the classification and naming of viruses that infect plants (Murphy *et al.*, 1995; Fauquet and Mayo, 1999; Fauquet *et al.*, 2000; Van Regenmortel *et al.*, 2000). The Executive Committee has recommended that approved geminivirus species (Table 1-4) should be classified into one of four separate genera of the family *Geminiviridae.* The leafhopper-transmitted viruses have been grouped into either the genus *Mastrevirus* (Table 1) or *Curtovirus* (Table 2) in accordance with the type of plant host and genome arrangement. All of the whitefly-transmitted viruses are grouped into the genus *Begomovirus* (Table 4) (Briddon and Markham, 1995). Recently, the new genus of *Topocuvirus* (Table 3) has been established to accommodate a known treehopper-transmitted virus (Pringle, 1999).

Traditionally, the names of plant viruses have been devised from descriptive information about the virus including its primary host, the most distinctive symptom induced in the infected plant, and the geographic location where the virus was first found. The naming of new viruses has become confusing, as different viruses may cause similar symptoms in the same crop within the same geographic region. In order to overcome the limitations for naming viruses, it has been recommended that where numerous strains exist, the geographic location of the species first found should be added within the core of the name of the virus, e.g. *Tomato yellow leaf curl Sardinia virus* (Fauquet *et al.*, 2000). This proposed system allows greater freedom for the future and is consistent with the system used for naming animal viruses. Further, it has been proposed that the first published name of a virus should be retained without any modification. This system of nomenclature has been adopted throughout this text.

Table 1. Genus: *Mastrevirus* (formerly subgroup I geminiviruses). Virus species in normal type have been reported and those shown in italic type have been approved by the ICTV.

Species	Genome size (nucleotides)
Bean yellow dwarf virus (BeYDV)	2561
Bromus striate mosaic virus (BrSMV)	
Chloris striate mosaic virus (CSMV)	2750
Digitaria streak virus (DSV)	2701
Digitaria striate mosaic virus (DiSMV)	
Maize streak virus (MSV)	2681, 2682, 2683, 2685, 2687, 2690
Miscanthus streak virus (MiSV)	2672
Panicum streak virus (PanSV)	2700, 2705
Paspalum striate mosaic virus (PSMV)	
Setaria streak virus (SetSV)	2701
Sugarcane streak virus (SSV)	2704, 2706, 2707, 2735, 2758, 2785
Tobacco yellow dwarf virus (TYDV)	2580
Wheat dwarf virus (WDV)	2749 or 2750
Tentative members	
Bajra streak virus (BaSV)	
Chickpea chlorotic dwarf virus (CpCDV)	
Millet streak virus (MISV)	

Table 2. Genus: *Curtovirus* (formerly subgroup II geminiviruses).

Species	Genome size (nucleotides)
Beet mild curly top virus (BMCTV) [-Worland]	2930
Beet severe curly top virus (BSCTV) [-CFH]	2923, 2927, 2930
Beet curly top virus (BCTV) [-California and –Logan]	2927, 2993, 2994, 2997
Horseradish curly top virus (HrCTV)	3080
Tentative member	
Tomato leafroll virus (TLRV)	

Table 3. Genus *Topocuvirus.*

Species	Genome size (nucleotides)
Tomato pseudo curly top virus (TPCTV)	2861

Table 4. Genus: *Begomovirus* (formerly subgroup III geminiviruses). Virus species in normal type have been reported and those shown in italic type have been approved by the ICTV.

Species	Genome size (nucleotides)
Abutilon mosaic virus (AbMV)	A = 2629, 2634; B = 2581, 2571
African cassava mosaic virus (ACMV)	A = 2777, 2779, 2780, 2781-2, 2750; B = 2726, 2724, 2720, 2725, 2726
Ageratum yellow vein virus (AYVV)	A = 2741, 2734; B = 1347
Althea rosea enation virus (AREV)	A = 2755
Bean calico mosaic virus (BCaMV)	A= 2603; B= 2572
Bean dwarf mosaic virus (BDMV)	A = 2615; B = 2576
Bean golden mosaic virus (BGMV)	A = 2585-2647; B = 2580-2647
Bean golden yellow mosaic virus (BGYMV)	A = 2646, 2585; B = 2587, 2647
Cabbage leaf curl virus (CaLCuV)	A = 2583; B = 2507
Chayote mosaic virus (ChaMV)	A = 2787
Cotton leaf crumple virus (CLCrV)	A = 2750
Cotton leaf curl Alabad virus (CLCuAV)	A = 2744
Cotton leaf curl Kokhran virus (CLCuKV)	A = 2751
Cotton leaf curl Multan virus (CLCuMV)	A = 2748, 2758, 2722,
Cowpea golden mosaic virus (CPGMV)	A = 2728
Cucurbit leaf crumple virus (CuLCrV)	A = 2632; B = 2600
Croton yellow vein mosaic virus (CYVMV)	
Dicliptera yellow mottle virus (DiYMoV)	A = 2607; B = 2597
East African cassava mosaic virus (EACMV)	A = 2750–2804; B = 2741-2777
Eupatorium yellow vein virus (EpYVV)	A = 2766, 2765
Euphorbia mosaic virus (EuMV)	
Honeysuckle yellow vein mosaic virus (HYVMV)	A = 2757
Indian cassava mosaic virus (ICMV)	A = 2815; B = 2645
Jatropha mosaic virus (JMV)	
Leonurus mosaic virus (LeMV)	
Macroptillium golden mosaic virus (MGMV)	
Mungbean yellow mosaic Indian virus (MYMIV)	A = 2745, 2750, 2723; B = 2616, 2656, 2675
Mungbean yellow mosaic virus (MYMV)	A = 2723, 2725, 2726; B = 2675
Okra enation virus (OkEV)	A = 2764
Okra yellow vein mosaic virus (OYVMV)	A = 2739, 2741; B = 2739
Papaya leaf curl virus (PaLCuV)	A = 2746
Pepper golden mosaic virus (PepGMV)	A = 2613, 2619
Pepper huasteco yellow vein virus (PHYVV)	A = 2631, B = 2589
Pepper leaf curl virus (PepLCV)	A = 2744
Potato yellow mosaic virus (PYMV)	A = 2582-2593, B = 2535-2547
Pseuderanthemum yellow vein virus (PYVV)	
Rhynchosia golden mosaic virus (RhGMV)	A = 2624
Sida golden mosaic Costa Rica virus (SiGMCRV)	A = 2605; B = 2587
Sida golden mosaic Florida virus (SiGMFV)	A = 2642; B = 2585
Sida golden mosaic Honduras virus (SiGMHNV)	A = 2603, 2612; B = 2589, 2593, 2569, 2593
Solanum tomato leaf curl virus (SToLCV)	
South African cassava mosaic virus (SACMV)	A = 2800; B = 2758, 2760

Begomoviruses (continued)	Genome size (nucleotides)
Soybean crinkle leaf virus (SbCLV)	
Squash leaf curl China virus (SLCCV)	A = 2741
Squash leaf curl virus (SLCV)	A = 2634, 2635; B = 2607, 2604,
Squash yellow mottle virus (SYMoV)	
Sweet potato leaf curl virus (SPLCV)	A = 2828, 2750
Tobacco apical stunt virus (TbASV)	
Tobacco leaf curl China virus (TbLCCNV)	A = 2746
Tobacco leaf curl India virus (TbLCIV)	
Tobacco leaf curl virus (TbLCV)	
Tobacco leaf curl Japan virus (TbLCJV)	A = 2766, 2761
Tobacco leaf curl Yunan virus (TbLCYV)	A = 2747
Tomato curly stunt virus (TCuSV)	
Tomato dwarf leaf curl virus (ToDLCV)	
Tomato golden mosaic virus (TGMV)	A = 2588, B = 2508, 2524
Tomato golden mottle virus (TGMoV)	
Tomato leaf crumple virus (ToLCrV)	A = 2624-2634; B = 2576-2627
Tomato leaf curl Bangalore virus (ToLCBV)	A = 2749
Tomato leaf curl Bangalore virus (ToLCBV-[Ban4]	A = 2759
Tomato leaf curl Bangladesh virus (ToLCBDV)	A = 2761
Tomato leaf curl India virus (ToLCIV)	
Tomato leaf curl Indonesia virus (ToLCIDV)	
Tomato leaf curl Karnataka virus (ToLCKV)	A = 2759
Tomato leaf curl Laos virus (ToLCLV)	A = 2748
Tomato leaf curl New Delhi virus (ToLCNdV)	A = 2739-2750; B = 2696
Tomato leaf curl Nicaragua virus (ToLCNV)	
Tomato leaf curl Philippine virus (ToLCPV)	
Tomato leaf curl Senegal virus (ToLCSV)	
Tomato leaf curl Sinaloa virus (ToLCSinV)	
Tomato leaf curl Sri Lanka virus (ToLCSLV)	A = 2756
Tomato leaf curl Taiwan virus (ToLCTWV)	A = 2739
Tomato leaf curl Tanzania virus (ToLCTZV)	
Tomato leaf curl virus (ToLCV)	A = 2766, 2767
Tomato leaf curl Yunan virus (ToLCYV)	
Tomato mild mottle virus (ToMMoV)	
Tomato mosaic Barbados virus (ToMBV)	
Tomato mosaic Havana mosaic virus (ToMHV)	A = 2620; B = 2586
Tomato mottle Taino virus (ToMoTV)	A = 2597; B = 2562
Tomato mottle virus (ToMoV)	A = 2601; B = 2544
Tomato rugose mosaic virus (ToRMV)	A = 2622; B = 2572
Tomato severe leaf curl virus (ToSLCV)	
Tomato Uberlandia virus (ToUV)	
Tomato yellow dwarf virus (ToYDV)	
Tomato yellow leaf curl China virus (TYLCCV)	A = 2734
Tomato yellow leaf curl Kuwait virus (TYLCKWV)	
Tomato yellow leaf curl Sardinia virus (TYLCSV)	A = 2773
Tomato leaf curl Soudan virus (TYLCSDV)	
Tomato yellow leaf curl Thailand virus (TYLCTHV)	A = 2743, 2751; B = 2737, 2737
Tomato yellow leaf curl virus (TYLCV)	A = 2787

Begomoviruses (continued)	Genome size (nucleotides)
Tomato yellow leaf curl Yemen virus (TYLCYV)	
Tomato yellow mottle virus (ToYMoV)	
Tomato yellow vein streak virus (ToYVSV)	
Watermelon chlorotic stunt virus (WmCSV)	A = 2752, 2753; B = 2728, 2760
Wissadula golden mosaic virus (WGMV)	
Tentative members	
Eggplant yellow mosaic (EYMV)	
Lupin leaf curl virus (LLCV)	
Sida yellow vein virus (SiYVV)	
Solanum leaf curl virus (SALCV)	

1.3 Genome organisation of *Geminiviridae*

The original reports of viruses have used differing systems for describing the organisation of their genomes. In the interest of uniformity and clarity, the naming of open reading frames recommended by the ICTV (Figure 2) has been adopted throughout this text. All genomic DNAs of geminiviruses contain divergent coding sequences separated by intergenic regions. The mapping of transcripts has shown that the viral genes are located on both the virion-sense (V) and complementary-sense (C) DNA strands (Morris-Krsinich *et al.*, 1985; Townsend *et al.*, 1985; Accotto *et al.*, 1989; Sunter and Bisaro, 1989; Sunter *et al.*, 1989; Dekker *et al.*, 1991; Frischmuth *et al.*, 1991; Mullineaux *et al.*, 1993).

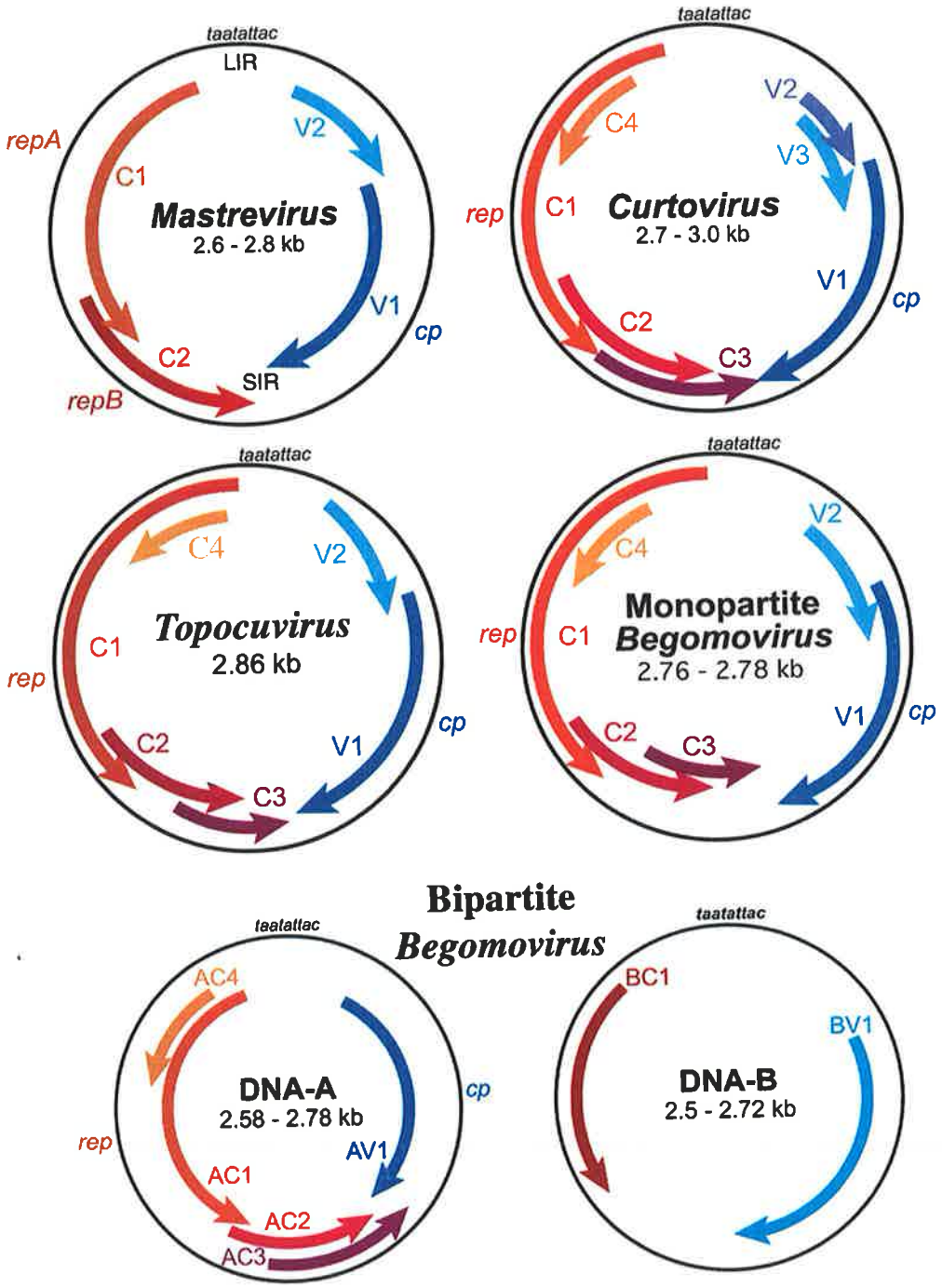


Figure 2. The genome organisation of *Geminiviridae* (adapted from Van Regenmortel *et al.*, 2000) showing the open reading frames (arrows), the position of the large intergenic region (LIR) containing a conserved stem loop sequence (*taatattac*), and the small intergenic region (SIR). The location of the essential replication initiator gene (*rep/repA-repB*) and coat protein gene (*cp*) are also indicated.

1.3.1 Mastrevirus

Mastreviruses are transmitted in nature by leafhoppers (Homoptera: *Cicadellidae*) in a circulative and persistent manner to a narrow plant host range of mostly grasses (Bock, 1974). *Tobacco yellow dwarf virus* (TYDV) and *Bean yellow dwarf virus* (BeYDV) differ from the monocot-infecting members by infecting dicotyledonous plants. Serological analyses indicate that mastreviruses from different continents are either distantly related or unrelated with some species reported to be spread naturally within Africa, Australia, Europe, India, Pakistan, Russia, and Vanuatu. The distinctive serological groupings include:

- African streak group (MSV, PanSV, SSV)
- Australian striate mosaic group (CSMV, BrSMV, DiSMV, PSMV)
- MiSV (Japan)
- WDV (Europe, Middle East)
- BeYDV (South Africa)
- DSV (Vanuatu)
- TYDV (Australasia)

The monopartite genome of mastreviruses (MacDowell *et al.*, 1985; Donson *et al.*, 1987; Lazarowitz, 1988) illustrated in Figure 2 ranges in size from 2.56 to 2.78 kb (Table 1). Their genome contains four open reading frames (ORFs) with V1 adjacent to or overlapping V2 on the virion-sense strand and C1 overlapping C2 on the complementary-sense strand. The C1 and C2 ORFs form the *repA* and *repB* exons of the essential replication associated protein (Rep) (Schalk *et al.*, 1989; Mullineaux *et al.*, 1990; Dekker *et al.*, 1991; Wright *et al.*, 1997). The large intergenic region (LIR) contains the origin of replication and the virion- and complementary-sense promoters. The small intergenic region (SIR) contains the bi-directional polyadenylation signals.

The host range information of *Maize streak virus* (MSV) including its properities for determining the severity of chlorosis, streak length, timing of symptom appearance have been mapped to a viral fragment that spans the large intergenic region and the 5' terminus of the complementary sense C1 ORF (Boulton *et al.*, 1991a; Boulton *et al.*, 1991b). The streak width character appears to be determined by the virion-sense portion of the genome which contains the viral coat protein gene (V1), that in combination with ORF V2, mediates viral movement (Morris-Krsinich *et al.*, 1985; Boulton *et al.*, 1993). Mutations in both ORFs prevented systemic infection and reduced the accumulation of single-stranded DNA in infected cells (Lazarowitz *et al.*, 1989; Woolston *et al.*, 1989; Boulton *et al.*, 1993).

1.3.2 Curtovirus

Beet curly top virus (BCTV) is the type member of this genus and infects a wide range of plant hosts including some 300 species in 44 plant families (Bennett, 1971). BCTV is transmitted in a circulative and persistent manner by the leafhopper *Circulifer tenellus* (Homoptera: *Cicadellidae*) in North America and by both *C. tenellus* and *C. opacipennis* in the Mediterranean Basin (Thomas and Mink, 1979). Beet curly top disease also spreads in the African region, the Eurasian region and in Central and South America. Several isolates of BCTV have been characterised and found to have a monopartite genome consisting of a single DNA component ranging from 2.92 to 3.08 kb (Table 2). The BCTV genome is known to encode seven genes with three ORFs on the virion-strand (Figure 2). The V1 (coat protein) and V3 (movement protein) are both required for systemic viral infection whereas the product of the V2 ORF appears to modulate the conversion of single-stranded DNA (ssDNA) to double-stranded DNA (dsDNA) (Briddon *et al.*, 1989; Stanley *et al.*, 1992; Frischmuth *et al.*, 1993; Hormuzdi

and Bisaro, 1993). Four overlapping ORFs (C1-4) are present on the complementary-sense strand. The C1 (Rep) is needed for the replication of viral DNA and the C3 ORF product enhances the replication or the accumulation of viral DNA. The C4 ORF appears to be involved in pathogenesis (Briddon *et al.*, 1989; Stanley and Latham, 1992; Stanley *et al.*, 1992; Frischmuth *et al.*, 1993; Hormuzdi and Bisaro, 1993). The role of the C2 remains unclear although its ORF appears to be functional (Stanley *et al.*, 1992).

Horseradish curly top virus (HrCTV) is a virus that may have arisen from a recombinant event between a BCTV-like ancestor and a begomovirus-like ancestor. Analyses of the HrCTV genome reveal that its V1, V2 and V3 virion-sense ORFs are more closely related to the corresponding ORFs of BCTV whereas its complementary-sense ORFs (C1, C2 and C4) differ from the BCTV model by lacking the C3 ORF. Further, the C1 and C4 ORFs of HrCTV share closer phylogenetic relationships to the corresponding ORFs of the begomovirus SLCV.

1.3.3 Topocuvirus

Tomato pseudo curl top virus (TPCTV) is currently the sole member of the genus *Topocuvirus.* This virus has a narrow host range and is transmitted by the treehopper *Micrutalis malleifera* (Hemiptera: Membracidae) in a semi-persistent manner to solaneous plants in Florida, USA (http://life.anu.edu.au/viruses/ICTVdb/). TPCTV has a genome organisation similar to BCTV and monopartite begomoviruses (Briddon *et al.*, 1996).

1.3.4 Begomovirus

This genus comprises viral members with either bipartite or monpartite genomes that are transmitted by whiteflies of the *Bemisia* species (*Aleyrodidae*) in a persistent-circulative way to dicotyledonous plants (Zeidan and Czosnek, 1991; Markham *et al.*, 1994). The DNA-A and DNA-B molecules of the bipartite viruses are both required for efficient infection (Stanley and Gay, 1983; Hamilton *et al.*, 1983; Hamilton *et al.*, 1984; Howarth *et al.*, 1985; Stanley and Townsend, 1986; Etessami *et al.*, 1988; Frischmuth *et al.*, 1990; Klinkenberg and Stanley, 1990; Evans and Jeske, 1993; Padidam *et al.*, 1995a; Sung and Coutts, 1995). The total genome size of a bipartite begomovirus ranges between 4.09 and 5.61 kb (Table 4).

Depending upon the virus, the DNA-A component has four or five ORFs. The ORF AV1 on the virion-sense strand is the coat protein gene (Gardiner *et al.*, 1988; Kallender *et al.*, 1988; Briddon *et al.*, 1990; Sunter *et al.*, 1990; Sung and Coutts, 1995; Pooma *et al.*, 1996; Hofer *et al.*, 1997). The New Delhi isolate of ToLCV (ToLCNdV) has an additional AV2 ORF which provides movement function and affects the accumulation of both single-stranded and double-stranded DNA (Padidam *et al.*, 1996). This ORF is not present in the viruses from the Americas. Bipartite begomoviruses have three functional ORFs on the complementary-sense strand encoding Rep (AC1), transactivation function (AC2), and an enhancer of DNA replication (AC3) (Elmer *et al.*, 1988; Hayes and Buck, 1989; Hanley-Bowdoin *et al.*, 1990; Sunter *et al.*, 1990; Etessami *et al.*, 1991; Morris *et al.*, 1991; Sunter and Bisaro, 1991; Sung and Coutts, 1995). The presence of a putative AC4 ORF is controversial as its function has not been clearly demonstrated or any effect observed on viral replication when this putative ORF of bipartite begomoviruses has been rendered defective by mutagenesis (Elmer *et*

al., 1988; Sung and Coutts, 1995; Pooma and Petty, 1996). The smaller DNA-B component has only two ORFs, with one each on the virion-sense and complementary-sense strands (BV1 and BC1) that both produce movement proteins (Noueiry *et al.*, 1994; Pascal *et al.*, 1994; Jeffrey *et al.*, 1996; Sanderfoot *et al.*, 1996; Ward *et al.*, 1997). Transgenic plants with stably integrated partial tandem copies of *Tomato golden mosaic virus* (TGMV) DNA-A demonstrated that the A-component alone carries all of the genetic information necessary for viral replication and encapsidation (Rogers *et al.*, 1986; Sunter *et al.*, 1987). A similar approach showed that the B-component could not replicate in the absence of DNA-A, but it was additionally required for systemic movement of the viruses and for symptom production (Brough *et al.*, 1988; Pascal *et al.*, 1993; Sanderfoot *et al.*, 1996).

The monopartite species have been shown to have a genome organisation similar to the *Curtovirus* model and the DNA-A component of bipartite begomoviruses. These monopartite viruses are able to encode all functions required for the complete infection cycle in their host plants (Kheyr-Pour *et al.*, 1991; Navot *et al.*, 1991; Dry *et al.*, 1993; Noris *et al.*, 1994). Their genome contains six ORFs (Figure 2), and ranges in size from 2.58 to 2.83 kb (Table 4). The virion DNA strands of monopartite begomoviruses are more complex than the virion-sense strands of bipartite members. The two partially overlapping ORFs of monopartite species encode a non-structural protein (V2) and the coat protein (V1). The expression of both ORFs is necessary to elicit disease (Rigden *et al.*, 1993; Padidam *et al.*, 1996; Wartig *et al.*, 1997). Three of the four ORFs located on the complementary-sense strand overlap partially, and the C1 ORF completely overlaps the C4 ORF. Like the bipartite members, the C1 ORF encodes for Rep, the C2 ORF appears to be involved in the regulation of dsDNA and ssDNA, through the *trans-*

activation of the coat protein gene (Dry *et al.*, 2000), and the C3 ORF may function as an enhancer of viral DNA replication. Unlike the bipartite members, the C4 ORF appears to be functional in all species examined so far and seems to be somehow involved in the development of disease symptoms and/or in systemic viral movement (Jupin *et al.*, 1994; Rigden *et al.*, 1994).

1.4 Replication and transcription of geminiviruses

The extraction of viral DNA from all geminivirus-infected plants has revealed the presence of supercoiled, open-circular and linear dsDNA forms. The dependency of geminiviruses on the plant host DNA and RNA polymerases for their replication and transcription is demonstrated by the fact that deproteinised viral ssDNA and RNA-free alkali-prepared DNA from mechanically-transmissible viruses (BGMV, TGMV, MYMV) remained infectious after treatment (Goodman, 1977a; Goodman, 1977b; Hamilton *et al.*, 1981; Hamilton *et al.*, 1982; Ikegami *et al.*, 1984). A body of evidence has built up to show that geminivirus genomes employ a rolling-circle replication mechanism to amplify their single-stranded genomes to produce double-stranded DNA forms that serve as templates for replication and transcription (Saunders *et al.*, 1991; Stenger *et al.*, 1991; Heyraud *et al.*, 1993a; Heyraud *et al.*, 1993b).

1.4.1 Replication of viral DNA

Replication of DNA by the rolling-circle mechanism is a two-step process in which the synthesis of leading and lagging-strands are separate events (Kornberg and Baker, 1992). During the first step, the single-stranded virion-sense strand ("plus strand") is used as a template for the synthesis of the complementary-sense strand ("minus strand") to generate a double-stranded replicative form. During the second step, the

replicative form serves as a template for plus-strand synthesis to generate free single-stranded DNA.

Replication of geminivirus DNA requires two origins, one for plus-strand replication and another for minus-strand synthesis. Rolling-circle mechanisms use a site-specific nick to prime the synthesis of plus-strand DNA. RNA generated through the activity of RNA polymerase or DNA primase is needed to prime synthesis of the minus strand. The plus-strand origin of viral members from all genera has been mapped to the 5' intergenic region that contains the promoters and controlling elements for virion- and complementary-sense transcription (Hanley-Bowdoin *et al.*, 1999). This region of all geminiviruses contains an inverted repeat that is capable of forming a hairpin structure with the conserved sequence 5'-TAATATTAC-3' within the loop of the hairpin. Although the overall sequence of the 5' intergenic region is not conserved between different viruses, it is nevertheless highly conserved (>95%) in the common intergenic regions of both the DNA-A and DNA-B components of bipartite begomoviruses. This homology indicates that this region contains recognition signals essential for processes involved in replication (Lazarowitz, 1992). Further, structural analysis of the intergenic region of a number of geminiviruses revealed the presence of iterated sequence motifs, which have been proposed to be specific binding sites of replication associated proteins (Arguello-Astorga *et al.*, 1994; Behjatnia *et al.*, 1998).

Studies have shown that geminivirus Rep is a site-specific endonuclease that nicks plus-strand viral DNA (Heyraud-Nitschke *et al.*, 1995; Laufs *et al.*, 1995b; Orozco and Hanley-Bowdoin, 1996). The initiation site of the plus-strand was precisely mapped *in vivo* to the conserved nonanucleotide sequence (TAATATT/AC) of the hairpin loop

(Stanley, 1995). Together, these studies demonstrate that plus-strand synthesis initiates within a conserved hairpin motif through the endonucleoytic action of Rep. The subsequent events involved in plus-strand DNA synthesis and enzymes that mediate them are not well characterised. The plant cell nuclear machinery is most likely responsible for the elongation phase of plus-strand DNA synthesis. The exception may be the source of the DNA helicase that catalyzes unwinding of the parental plus-strand.

During rolling-circle replication of the geminivirus genome, the Rep specifically binds to the intergenic region sequences of the double-stranded replicative DNA and induces a local melting and destabilisation of a cruciform extrusion of the hairpin at the origin of replication. This binding site is specific for each virus, which explains why the *rep* of related viruses are not interchangeable (Lazarowitz, 1992). Subsequently, the Rep cleaves the plus strand within the conserved sequence motif. The liberated 3' hydroxyl group of nucleotide +7 (thymidine) is available to prime the plus-strand DNA synthesis while the 5' end of the cleaved strand becomes covalently linked to the Rep and becomes displaced. Following a complete round of extension, the newly synthesised origin sequence is recognised and bound to the Rep that cleaves the newly synthesised sequence. Ligation of newly generated 3' hydroxyl group and the 5' end attached to Rep occurs via an energy-conserving ester bond to a consensus tyrosine and the release of the circular ssDNA of genomic length occurs (Desbiez *et al.*, 1995; Laufs *et al.*, 1995a). The circular ssDNA may enter into further DNA replication to serve as a template for a new generation of additional dsDNA, or it may be sequestered for the purpose of encapsidation. The regulation of this process seems to be under the control of coat protein production (Saunders *et al.*, 1991). Absence of coat protein in the early

phase of replication may lead DNA back into replication, whereas its presence in the latter stages of the infection cycle would lead to encapsidation.

Less is known about the mechanism and events leading to the synthesis of the minus-strand. It is generally believed that minus-strand DNA synthesis is mediated entirely by host factors (Hanley-Bowdoin *et al.*, 1999). In mastreviruses, a small DNA fragment resembling a primer-like oligonucleotide has been found annealed within the intergenic region of viral DNA in five members (MSV, CSMV, DSV, TYDV and WDV) and shown that it could be extended by DNA polymerase *in vitro* (Donson *et al.*, 1984; Hayes *et al.*, 1988a). It has been proposed that this oligonucleotide may function to prime the minus-strand *in vivo.* In addition, a sequence in the 3'-intergenic region of WDV has been implicated in replication and this region may be the minus-strand origin (MacDonald *et al.*, 1988; Kammann *et al.*, 1991). Analogous oligonucleotides have not been detected in virions of curtoviruses and begomoviruses (Hanley-Bowdoin *et al.*, 1999). In the case of ACMV, the synthesis of the minus-sense DNA on the plus-sense template has been reported to be preceded by the synthesis of an RNA primer (Saunders *et al.*, 1992). The presence of the ribonucleotide supports the probability of its involvement in priming minus-strand synthesis. This RNA could prime complementary-sense DNA synthesis on the circular ssDNA template. The double-stranded replicative form generated during the replication cycle would not only provide a template for the generation of viral-sense DNA but it could also interact with host factors to be converted into transcriptionally active supercoiled DNA. Further viral-sense ssDNA could then be produced from the dsDNA by rolling-circle replication (Saunders *et al.*, 1991).

1.4.2 Bi-directional transcription

Transcription of the viral genome occurs in the nucleus of the plant host cell and is performed by host cell enzymes. The viral messenger RNAs (mRNAs) have been characterised for several viruses (Townsend *et al.*, 1985; Petty *et al.*, 1988; Hanley-Bowdoin *et al.*, 1989; Sunter and Bisaro, 1989; Sunter *et al.*, 1989; Frischmuth *et al.*, 1991; Mullineaux *et al.*, 1993). These studies established that the genomes of geminiviruses are transcribed in a bi-directional manner resulting in mRNAs that span either virion or complementary-sense ORFs. The viral mRNAs are polyadenylated and initiate downstream of either TATA-box motifs or initiator elements, indicating that they are transcribed by host RNA polymerase II (Hanley-Bowdoin *et al.*, 1999). Transcription terminates at the polyadenylation site located in the 3'intergenic region where virion- and complementary-sense ORFs converge.

Transcription in geminiviruses produces multiple overlapping RNA species that may be monocistronic or polycistronic. The monocistronic RNAs produce a single protein from an ORF whereas the polycistronic RNAs span more than one ORF and proteins may be translated by a leaky scanning mechanism. In mastreviruses, the transcript spanning the C1-C2 ORFs contains an intron that after processing forms a single contiguous Rep coding sequence (Accotto *et al.*, 1989; Schalk *et al.*, 1989; Mullineaux *et al.*, 1990). More recently, another intron was identified in ORF V1 of MSV and DSV.

1.5 Function of the viral open reading frames (ORFs)

Temporal regulation of gene expression is needed to coordinate and regulate the different events that occur during the process of viral infection. Geminiviruses control the timing of their own gene expression through the concerted actions of the viral-

encoded proteins from the open reading frames that interact with viral genome and the host cell machinery. The viral products required for replication and regulation are expressed early in the infection cycle whereas gene products needed for viral movement and for encapsidation are produced later in the infection cycle.

Mastreviruses show temporal regulation over the expression of the viral strand genes with the early *c1-c2* product (Hofer *et al.*, 1992). As both the spliced transcript and the unspliced precursor of *c1-c2* are present in infected tissues, differential splicing may also regulate viral gene expression (Accotto *et al.*, 1989; Mullineaux *et al.*, 1990; Dekker *et al.*, 1991; Wright *et al.*, 1997). In begomoviruses such as TGMV and ACMV, the *ac1* gene can autoregulate its own expression (Haley *et al.*, 1992; Sunter *et al.*, 1993). Also, the promoters of bipartite viral genes that are required late in the infection cycle are relatively weak in transient assays (Zhan *et al.*, 1991), and are activated by an early gene product encoded by *ac2* (Sunter and Bisaro, 1991; Haley *et al.*, 1992; Sunter and Bisaro, 1992). A study of the promoters that control each of the six ORFs of ToLCV has indicated that the individual promoters are differentially regulated by viral and host factors, leading to distinct patterns of expression in host cells (Dry *et al.*, 2000).

1.5.1 Coat protein gene (V1 or AV1 ORF)

The viral coat protein (CP) is the most abundant viral product in plant tissues infected by geminiviruses. It is encoded from the virion-sense strand of monopartite viruses (Morris-Krsinich *et al.*, 1985) and the DNA-A component of bipartite viruses (Stanley and Townsend, 1985). The viral coat protein is a structural protein needed in the encapsidation process leading to the formation of mature virions that spread throughout

the plant. Additionally, coat protein serves an essential role in virus acquisition by insect vectors by protecting the viral DNA during transmission and as the determinant of insect vector specificity (Briddon *et al.*, 1990; Azzam *et al.*, 1994; Hofer *et al.*, 1997). Comparison of the coat protein sequence of whitefly-transmissible strains of AbMV with non-transmissible strains indicated that some or all of the 5 amino acids at the N-terminus of the coat protein are involved in the whitefly-transmission of bipartite begomoviruses (Wu *et al.*, 1996).

Monopartite viruses (MSV, WDV, BYDV, BCTV, ToLCV and TYLCSV) representing the *Mastrevirus, Curtovirus* and *Begomovirus* genera have been shown to require a functional coat protein gene (*cp*) for systemic viral invasion by virus and for the development of disease in their host plants. The *cp*-defective mutant viruses replicated in cells, although they produced lower levels of dsDNA than their equivalent wild-type viruses (Morris-Krsinich *et al.*, 1985; Boulton *et al.*, 1989; Briddon *et al.*, 1989; Lazarowitz *et al.*, 1989; Woolston *et al.*, 1989; Boulton *et al.*, 1993; Hormuzdi and Bisaro, 1993; Rigden *et al.*, 1993; Wartig *et al.*, 1997; Liu *et al.*, 1998).

In contrast, the *cp* gene of TGMV can be substantially interrupted or replaced before replication and cellular movement function of *cp*-defective mutants is affected in hosts such as *Nicotiana benthamiana* (Bisaro *et al.*, 1988; Brough *et al.*, 1988; Gardiner *et al.*, 1988; Hayes *et al.*, 1988a; Sunter *et al.*, 1990). These *cp*-defective viral mutants produced essentially normal levels of viral dsDNA in the infected cells but accumulated much lower levels of ssDNA than the wild-type virus. However, the extensively modified *cp*-deficient mutants of TGMV showed reproducible delays in the appearance of symptoms when inoculated into *N. benthamiana* plants containing the

TGMV DNA-B component. The most attenuated symptoms were obtained when the *cp* gene was almost entirely deleted (Gardiner *et al.*, 1988), and loss of infectivity occurred with a larger deletion (Brough *et al.*, 1988). Other virus-host combinations where the *cp* gene was not essential for systemic viral movement included BGMV in *Phaseolus vulgaris* (Azzam *et al.*, 1994), ToLCNdV in *Lycopersicon esculentum* (Padidam *et al.*, 1995b), and SLCV in *Cucurbita maxima* (Ingham *et al.*, 1995).

It has been shown that the ability of *cp*-defective mutants of bipartite begomoviruses to move systemically is determined by both viral and host factors (Pooma *et al.*, 1996). For instance, the *cp*-defective mutants of TGMV were shown not to systemically infect *N. tabacum* or *Datura stramonium* (Pooma *et al.*, 1996). Further, the *cp* gene of BGMV was required to allow systemic infection of *N. benthamiana* showing the nature of the host plant alone is not the only prerequisite for systemic viral invasion. Analysis of TGMV-BGMV hybrid viruses confirmed that the genetic background determined the dispensability of the *cp* gene for systemic viral movement (Pooma *et al.*, 1996). Conflicting information has been also reported for *cp*-defective mutants of ACMV in *N. benthamiana.* One report indicated that a DNA-A construct with a deleted *cp* gene induced the typical ACMV disease in *N. benthamiana* (Morris *et al.*, 1991), whereas another report concluded that systemic symptoms occurred only following reversion of the mutant to wild-type (Klinkenberg *et al.*, 1989).

It has been demonstrated that the coat protein of SLCV affected viral movement through its ability to influence the accumulation of the replicated viral ssDNA genomes (Qin *et al.*, 1998). A region of the coat protein was identified as binding to ssDNA and to be associated in the accumulation process of replicated viral ssDNA. These findings

indicate that the coat protein may act to signal the switch from viral dsDNA replication to the replication of viral ssDNA through the rolling circle mechanism or alternatively to sequester virion ssDNA from the replication pool without fully encapsidating it. (Qin *et al.*, 1998).

The mechanism of coat protein-mediated transfer of DNA from cell-to-cell and through the vascular bundle remains largely unknown. It has been suggested that binding of coat protein to MSV DNA may be involved in the nuclear transport of viral DNA, in addition to its function in the encapsidation process (Liu *et al.*, 1997). These studies showed that the coat protein of MSV bound both forms of viral DNA and plasmid DNA in a non-sequence specific manner. The binding domain was mapped to within the N-terminal 104 amino acids of the MSV coat protein. Also, the coat protein of TYLCV was shown to be a ssDNA binding protein which bound ssDNA in a highly cooperative and in a nonspecific-sequence fashion (Palanichelvam *et al.*, 1998). These CP-ssDNA complexes were resistant to nucleolytic digestion and remained stable at relatively high salt concentrations. Studies with the TYLCV-*cp* gene fused to the β-glucuronidase reporter enzyme in *Petunia hybrida* protoplasts and fluorescent-labelled-CP micro-injected into *Drosophila* embryos demonstrated that coat protein was transported into plant and insect cell nuclei by an active process of nuclear import via a nuclear localisation signal specific pathway (Kunik *et al.*, 1998). The TYLCV-CP nuclear localisation signal sequence was identified in the N-terminus of the protein.

Interactions between the *cp* gene and products of other ORFs of geminiviruses have been observed. A synergistic reduction in the level of ssDNA was obtained with a double V1-V2 ORF mutant of ToLCV, compared with corresponding levels obtained

with each single mutant (Rigden *et al.*, 1993), thus indicating that both V1 and V2 products play a dual role in the accumulation and conversion of viral ssDNA. Further, it has also been shown that the ability of *cp*-mutants of TGMV to accumulate ssDNA was dependent upon the function of the BV1 ORF (Jeffrey *et al.*, 1996). Also, the transcriptional activator protein encoded by the AC2 ORF of TGMV and ACMV has been shown to *trans*-activate the respective *cp* gene promoter and expression of the *bv1* gene (Sunter and Bisaro, 1991; Haley *et al.*, 1992; Sunter and Bisaro, 1992). It was further discovered that the AC2 product activated the TGMV *cp* gene promoter in *N. benthamiana* mesophyll cells but derepressed the promoter in phloem tissue (Sunter and Bisaro, 1997).

1.5.2 V2 and V3 ORFs

The V2 ORF of mastreviruses is required for infectivity and appears to participate with the coat protein gene in the mechanism for systemic viral movement and in the development of disease symptoms (Morris-Krsinich *et al.*, 1985; Boulton *et al.*, 1989; Lazarowitz *et al.*, 1989; Boulton *et al.*, 1991; Boulton *et al.*, 1993). BCTV requires V3 for systemic spread of the virus and the overlapping V2 ORF is involved in the process leading to either the synthesis or the regulation of ssDNA levels (Stanley *et al.*, 1992; Frischmuth *et al.*, 1993; Hormuzdi and Bisaro, 1993).

Studies indicate that both the coat protein and the non-structural protein encoded by the V2 ORF of ToLCV and TYLCSV participate in the regulation of ssDNA levels (Rigden *et al.*, 1993; Wartig *et al.*, 1997). It is possible that these proteins may sequester viral DNA. A mutant version of ToLCV with a dysfunctional V2 ORF reduced the yield of viral DNAs and induced a symptomless infection in tomato

(Rigden *et al.*, 1993). Similarly, a V2 mutant of the bipartite ToLCNdV from India developed extremely mild or no symptoms in *N. benthamiana* and accumulated lower levels of viral DNAs than the wild-type virus (Padidam *et al.*, 1996). Likewise, the V2 mutant versions of TYLCSV produced replication of dsDNA in *N. benthamiana* without disease symptoms but these mutants were unable to systemically invade tomato, indicating that this ORF played an essential role in the infection of tomato (Wartig *et al.*, 1997). The differing behaviour of the viral mutants may reflect a fundamental difference between ToLCV and TYLCV.

1.5.3 C1 (AC1) ORF

The product of the C1 or AC1 ORFs of curtoviruses and begomoviruses has been shown to form an essential replication associated protein (Rep) (Elmer *et al.*, 1988; Laufs *et al.*, 1995a; Hanley-Bowdoin *et al.*, 1999). Expression of Rep in transgenic plants has been shown to be sufficient to support the replication of replication-deficient mutants (Hanley-Bowdoin *et al.*, 1989; Hayes and Buck, 1989). The Rep of mastreviruses is formed from the *repA* and *repB* exons of the *c1-c2* transcript following the excision of the intron located within the overlapping region of the C1 and C2 ORFs. Viral mutants derived from mastreviruses with either a frameshift or deletion with the C1-C2 intron failed to replicate (Accotto *et al.*, 1989; Schalk *et al.*, 1989; Kammann *et al.*, 1991; Ugaki *et al.*, 1991).

Rep has been shown to be a multifunctional protein that performs key roles in geminivirus DNA replication and transcription (Laufs *et al.*, 1995a). The Rep interacts with the viral replication accessory factor AC3 (Settlage *et al.*, 1996), confers virus-specific recognition of its cognate origin of replication (Choi and Stenger, 1995; Jupin

et al., 1995; Gladfelter *et al.*, 1997), and initiates plus-strand DNA replication (Heyraud-Nitschke *et al.*, 1995; Laufs *et al.*, 1995b; Orozco and Hanley-Bowdoin, 1996). Also, Rep represses its own expression at the level of transcription (Sunter *et al.*, 1993; Eagle *et al.*, 1994) and may activate virion-sense transcription of some mastreviruses (Hofer *et al.*, 1992; Zhan *et al.*, 1993). In addition, the Rep of TGMV is involved in reactivating the cell division cycle in mature differentiated plant cells by inducing the expression of proliferating cell nuclear antigen (PCNA) which associates with host DNA polymerases to promote processivity (Nagar *et al.*, 1995). Similarly, it was also revealed that Rep of mastreviruses may reactivate the host DNA replication machinery through its interaction with a plant retinoblastoma factor (Xie *et al.*, 1995; Ach *et al.*, 1997). The LXCXE motif was shown to be functional in the Rep of WDV (Xie *et al.*, 1995), but this motif was not identified in curtoviruses or begomoviruses, indicating that another amino acid motif may interact with retinoblastoma-like proteins (Hanley-Bowdoin *et al.*, 1999).

The Rep of geminiviruses contains three conserved motifs that appear similar to the initiator proteins found associated with rolling circle replication systems in eubacterial plasmids (Ilyina and Koonin, 1992; Koonin and Ilyina, 1992). Interestingly, it has been shown that greater than unit length of the ToLCV genome can replicate in *Agrobacterium* dependent upon a functional Rep coding sequence, indicating that geminiviruses may have evolved from bacterial replicons (Rigden *et al.*, 1996). Studies have been aimed towards the identification of amino acids and domains that mediate the functions of Rep. The N-terminus of Rep appears to be responsible for the site-specific ssDNA cleavage and ligation function *in vitro* (Heyraud-Nitschke *et al.*, 1995; Laufs *et al.*, 1995a; Laufs *et al.*, 1995b; Orozco *et al.*, 1997). This region contains the

conserved motifs I (FLTY), II (HLH), and III (YXXK) that are associated with other initiator proteins (Ilyina and Koonin, 1992; Koonin and Ilyina, 1992). The N-termini of Rep from dicot-infecting geminiviruses also contain two sets of conserved α-helices that display limited sequence to known helices which may catalyze DNA unwinding (Gorbalenya and Koonin, 1993). In addition, the dsDNA binding, oligomerisation and AC3 interaction domains of TGMV Rep also map to the N-terminal half of the protein (Orozco *et al.*, 1997; Hanley-Bowdoin *et al.*, 1999). To date, ATP hydrolysis is the only biochemical activity attributed to the C-terminus (Desbiez *et al.*, 1995; Orozco *et al.*, 1997). The motif ($GXXXXGKT_{230}$) corresponding to a loop of a phosphate binding site that binds or hydrolyzes NTPs is conserved (Gorbalenya and Koonin, 1989). It is possible that the Rep C-terminus may also mediate other protein interactions including ssDNA binding, nuclear localisation and/or attachment to the nuclear matrix (Hanley-Bowdoin *et al.*, 1999).

1.5.4 C2 (AC2) ORF

The *ac2* gene of the bipartite begomoviruses TGMV and ACMV encodes a transcriptional activator which regulates the expression of the *av1* and *bv1* genes (Sunter and Bisaro, 1991; Haley *et al.*, 1992; Sunter and Bisaro, 1992). The AC2-defective mutant viruses did not induce systemic infection in plants, failed to produce viral coat protein and accumulated reduced amounts of ssDNA (Elmer *et al.*, 1988; Etessami *et al.*, 1991; Hanley-Bowdoin *et al.*, 1989; Hayes and Buck, 1989; Sunter *et al.*, 1990). The existence of a *cis*-acting repressor element located either within or near the C2 ORF of TGMV that represses coat protein promoter expression in the absence of the transcriptional activator AC2 protein has been suggested (Sunter and Bisaro,

1997). Recently *cis*-acting elements involved in the *trans*-activation of *cp* and *bv1* gene promoters of PHYVV have been identified (Ruiz-Medrano *et al.*, 1999).

The AC2 protein of PYMV was shown to bind ssDNA and dsDNA with high and low affinity, respectively, but in a non sequence-specific manner (Sung and Coutts, 1996). The amino acid sequence of AC2 of TGMV shows typical features of a DNA-binding protein, including a basic domain, zinc finger-like motif and an acidic domain (Sunter and Bisaro, 1992; Timmermans *et al.*, 1994). The zinc finger-like domain of 25 amino acid residues is the most conserved AC2 segment of begomoviruses. Ruiz-Medrano *et al.*, (1999) showed that this domain was also present in the C2 protein of TPCTV and strain CFH of BCTV (BSCTV), but not in either of the Logan and Californian strains of BCTV (BCTV-Cal) or the Worland strain of BCTV (BMCTV). The C2 protein of TYLCV was also shown to have DNA-binding activity *in vitro,* with preference to bind ssDNA in a non sequence-specific manner relative to dsDNA (Noris *et al.*, 1996). Studies with deletion mutants revealed that the central core of the C2 protein (amino acids 33-104) which contains a Cys-His rich region is sufficient to confer DNA binding activity.

The role of the C2 ORF in curtoviruses remains unclear. Studies with ORF C2-defective mutants of the Californian isolate and Logan strain of BCTV that had either been truncated by the introduction of termination codons or altered by frame-shifting were shown to be infectious and induced symptoms in both *N. benthamiana* and *Beta vulgaris* (Stanley *et al.*, 1992; Hormuzdi and Bisaro, 1995). Neither of these studies provided evidence for functional homology between the *c2* gene of BCTV and *ac2* of bipartite begomoviruses.

Little has been resolved on the actual role of the C2 ORF in the infection cycle of monopartite begomoviruses. It was demonstrated that a C2 mutant frame-shift version of TYLCSV prevented systemic viral infection of tomato but allowed attenuated infection of *N. benthamiana* (Wartig *et al.*, 1997). This mutation did not affect the ability to accumulate ssDNA and dsDNA in protoplasts and in *N. benthamiana* leaf discs. Also, western blot analysis showed that leaf discs inoculated with the C2-mutant virus contained a high level of coat protein, thus not supporting the hypothesis that the TYLCSV C2 gene product *trans*-activates the coat protein gene, unless this mechanism only occurs in specific combinations of virus and plant host. The absence of infectivity of the TYLCSV C2-mutant in tomato plants indicates a role for the C2 product in systemic spread of this virus in tomato. In contrast, comparison of replicative dsDNA and ssDNA levels obtained from ToLCV and mutant versions made with *c2*-defective and *v1,v2*-defective genes indicate that C2 may have a role in the *trans*-activation of ToLCV virion-sense genes (Dry *et al.*, 1997). Additional expression studies of gene fusions with the β-glucuronidase reporter gene further supported this role (Dry *et al.*, 2000). In mastreviruses, the C1-C2 protein may also act as a transcriptional activator. A mutant version of WDV with a nonfunctional C2 ORF was unable to replicate systemically and showed lowered activity of a promoter on the virion-sense strand (Hofer *et al.*, 1992).

1.5.5 C3 (AC3) ORF

Limited information is available on the function and biochemical properties of C3 or AC3 protein. Transfection of tobacco protoplasts with viral mutants of TGMV indicated that the AC3 ORF affected the efficiency of viral DNA replication (Sunter *et al.*, 1990). Subsequently, the *ac3* gene of ACMV was shown to interact with the

replication process (Etessami *et al.*, 1991). In this latter study, viral mutants with a defective-*ac3* gene accumulated 50-times less viral DNA in the infected plant tissues compared with wild-type virus under the same conditions. Further, it was shown that the mutation of *ac3* of ACMV caused a reduction in the level of its DNA-B component and reduced the severity of disease symptoms (Morris *et al.*, 1991). Although it seems that an intact *ac3* is required for the efficient replication of DNA-B, the exact nature of *ac3* interaction within the bipartite genome remains unknown.

The AC3 protein of TGMV has been found located in the nuclei of infected plant cells at levels similar to that of the Rep (Pedersen and Hanley-Bowdoin, 1994; Nagar *et al.*, 1995), indicating that it may function with AC1 during initiation of viral DNA replication (Hanley-Bowdoin *et al.*, 1999). The mechanism by which AC3 enhances viral DNA accumulation may reside in its ability to interact with Rep (Hanley-Bowdoin *et al.*, 1999). The AC3 protein shows no homology to any known enzymatic motifs, to date. Thus, it is possible that the structure of the AC3-Rep complex is important for replication rather than some other unknown catalytic activity of AC3 that affects Rep (Hanley-Bowdoin *et al.*, 1999). The C3 ORF of BCTV may also have a role in the regulation of viral DNA in some host plants (Stanley *et al.*, 1992). A mutant virus with a C3-defective ORF attenuated the symptoms in *Beta vulgaris* when compared with wild-type infection, whereas by contrast, no differences were observed between mutant and wild-type viruses in *N. benthamiana.*

1.5.6 C4 (AC4) ORF

Strains of BCTV cause the development of tumorous enations in the infected plants by inducing division of phloem parenchyma cells (hyperplasia). Comparison of symptoms

induced by wild-type BCTV and mutant versions with termination codons introduced into the C4 ORF, led to the suggestion that the product of the C4 ORF may be a major determinant of pathogenesis causing the hyperplastic response in BCTV-infected plants (Stanley and Latham, 1992; Stanley *et al.*, 1992). Although the mutant versions of BCTV induced stunting and yellowing in *N. benthamiana,* none of the vein-swelling or upward curling symptoms of wild-type infection occurred. The mutant viruses also caused symptomless infections in *Beta vulgaris*, contrasting with the stunting, vein-swelling and leaf curl symptoms associated with wild-type viral infection. In addition, the mutant viruses attenuated the induced disease symptoms in the inoculated *N. clevelandii* and *Datura stramonium* plants.

A similar stop-mutation in the C4 ORF of TYLCSV produced a viral mutant that attenuated the disease symptoms in *N. benthamiana* plants but generally this viral mutant failed to systemically infect tomato (*Lycopericon esculentum* var. Monique) (Jupin *et al.*, 1994). Analysis of the few infected tomato plants revealed that reversion of the mutation had occurred that restored the integrity of the C4 ORF, allowing the restored TYLCSV to cause systemic infection. The differing susceptibility of *N. benthamiana* and tomato indicated the interaction of other factors. The high degree of infectivity induced in *N. benthamaniana* (87%) and reduced viral DNA levels indicated that the resultant infection was not solely due to reversion of the mutant virus to the wild-type state in this host. It was suggested that the mild symptoms induced by the viral DNA with a mutated C4 ORF were caused by interaction between a mixture of mutant and reverted viral molecules. The authors concluded that the C4 ORF of TYLCSV encoded a protein involved in viral movement (Jupin *et al.*, 1994). In contrast, studies with a mutant C4 ORF version of ToLCV indicated that the C4 ORF

encoded a polypeptide that was not required by ToLCV for replication or systemic spread in three plant hosts tested (Rigden *et al.*, 1994). In this case, mutation of the C4 ORF of ToLCV to prevent the initiation of the C4 product reduced the severity of symptoms (Figure 3) induced in *D. stramonium, N. tabacum* and tomato (*Lycopericon esculentum* var. Grosse Lisse) while retaining wild-type levels of viral DNA species (Rigden *et al.*, 1994). In a few cases, reversion of the mutation back to the wild-type state was observed, but these plants all developed symptoms indistinguishable from plants inoculated with wild-type virus (Rigden *et al.*, 1994). These observations indicated that the C4 ORF encoded a polypeptide involved in symptom development, consistent with the role of the C4 ORF in BCTV suggested by Stanley and Latham (1992).

The evidence to date indicates that the AC4 ORF of bipartite *Begomovirus* species may be non-functional. Mutant versions of TGMV, ACMV, BGMV, and PYMV, carrying a disabled AC4 ORF, did not influence the efficiency of viral DNA replication or the nature of the induced phenotypic response in plants (Elmer *et al.*, 1988; Etessami *et al.*, 1991; Sung and Coutts, 1995; Hoogstraten *et al.*, 1996; Pooma and Petty, 1996). These studies indicated that the AC4 ORF of these viruses was non-functional in the hosts tested and may indicate that a redundant ORF is preserved because it overlaps the essential AC1 ORF (Etessami *et al.*, 1991; Pooma and Petty, 1996).

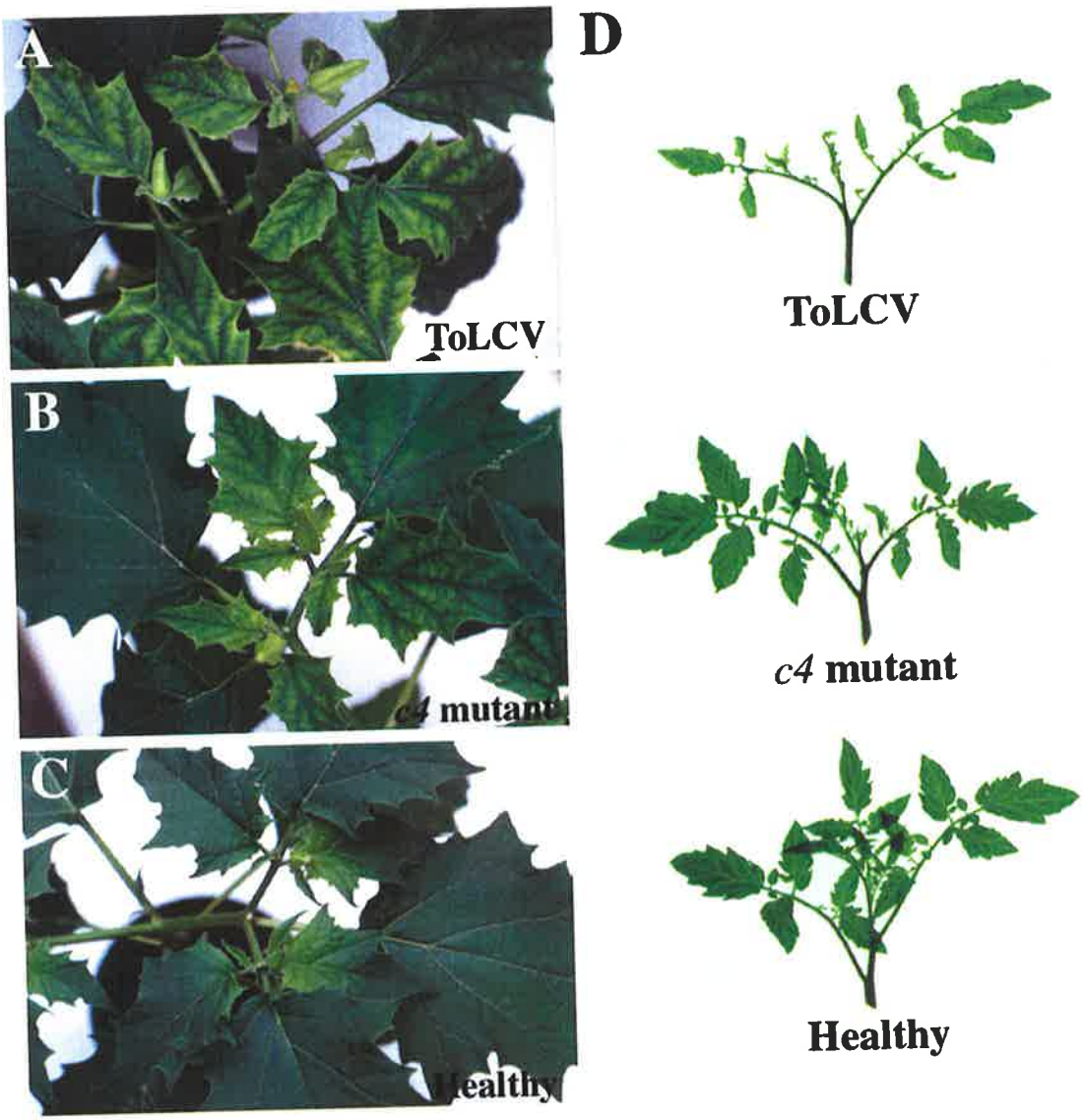


Figure 3. Comparison of symptoms induced in *Datura stramonium* by ToLCV (A), a C4 defective mutant of ToLCV (B) and a healthy plant (C) and a comparison of symptoms induced in tomato (D) by the native ToLCV and the mutant virus (extracted from studies reported by Rigden *et al.*, 1994).

1.5.7 BV1 and BC1 ORFs

Mutational analysis of TGMV indicated that the *bc1* gene was involved in cell-to-cell spread of the virus (Hayes and Buck, 1989). This gene codes for amino acid sequences

which share similarity to other known cell-to-cell movement proteins of various plant viruses (Koonin *et al.*, 1991). Since geminiviruses replicate in the nucleus, they face the challenge of movement into and out of the nucleus in addition to movement across the cell boundries. Bipartite begomoviruses utilise two DNA-B encoded proteins (BV1 and BC1) to coordinate nuclear and plasmodesmatal movement (Pascal *et al.*, 1993; Noueiry *et al.*, 1994; Pascal *et al.*, 1994; Ingham *et al.*, 1995; Sanderfoot and Lazarowitz, 1995; Sanderfoot *et al.*, 1996; Ward *et al.*, 1997; Rojas *et al.*, 1998).

The BV1 protein of SLCV has been found localised in the nuclei of phloem parenchyma and companion cells of systemically infected leaves of *Cucurbita maxima* and also to target the nucleus when transiently expressed in tobacco protoplasts (Pascal *et al.*, 1994; Sanderfoot and Lazarowitz, 1995). This BV1 product serves as a nuclear shuttle protein to bind the replicated viral ssDNA genome and transports it between the nucleus and the cytoplasm (Pascal *et al.*, 1994; Sanderfoot and Lazarowitz, 1995; Sanderfoot *et al.*, 1996; Ward and Lazarowitz, 1999).

The BC1 of SLCV functions at the plasma membrane and cell wall to facilitate the movement of BV1-ssDNA complexes to the adjacent phloem cells and sieve elements for long distance movement (Pascal *et al.*, 1993; Pascal *et al.*, 1994; Ingham *et al.*, 1995; Sanderfoot *et al.*, 1996). It appears that SLCV is able to recruit the endoplasmic reticulum of developing phloem cells as a conduit for cell-to-cell movement of the viral genome (Ward *et al.*, 1997). The BC1 protein was localised to unique tubules associated with systemically infected plants and none of these tubules were present in either healthy plants or in plants inoculated with SLCV *bc1*-defective viral mutants. It has been suggested that the BC1 protein associated with the endoplasmic reticulum-

derived tubules traps the BV1-ssDNA complexes in the cytoplasm and guides these along the tubules through the cell wall into adjacent cells (Ward *et al.*, 1997). When transported to adjacent cells, the BV1-ssDNA complexes would be released and targeted to the nucleus where a new round of replication and infection would be initiated (Lazarowitz and Beachy, 1999).

The BC1 protein of BDMV differs from BC1 of SLCV in its ability to bind nucleic acids (Pascal *et al.*, 1993; Ward *et al.*, 1997). The demonstration that BC1 of BDMV could bind viral dsDNA (Noueiry *et al.*, 1994), indicated that it may behave similarly to the *Tobacco mosaic virus* (TMV) movement protein by increasing the size exclusion limits of plasmodesmata. A further study of BDMV BV1 and BC1 DNA binding properties showed that they were able to recognise form-specific DNA in a size-specific manner that may provide the basis for the maintenance of genome size during systemic infection of plants (Rojas *et al.*, 1998). Preferential binding of BV1 with open-circular ssDNA and dsDNA forms and BC1 with open-circular dsDNA was observed. It appears that both ssDNA and dsDNA forms may be involved in cell-to-cell spread of BDMV. Whereas BDMV BV1 has a similar preference for binding both ssDNA and dsDNA, the BV1 of SLCV had been shown to preferentially bind ssDNA and BC1 of SLCV bound only weakly to ssDNA and not at all to dsDNA (Pascal *et al.*, 1994). These characteristics may reflect that SLCV is a phloem-restricted virus whereas BDMV is not restricted to any specific tissue of its host (Hoefert, 1987; Pascal *et al.*, 1994; Wang *et al.*, 1996; Sudarshana *et al.*, 1998).

Analyses of the BV1 and BC1 amino acid sequences and protein structures indicate the presence of positively-charged N-terminal domains that have the potential to interact

with DNA and hydrophobic domains which reflect a capacity for protein-protein interactions (Rojas *et al.*, 1998). It is proposed that BV1 and BC1 bind DNA via their positive-charged domains, obviating the need for sequence specificity. Alternatively, the binding of BV1 or BC1 to DNA could be mediated by short random nucleotide sequences, which would be present at higher frequencies on larger-sized DNA fragments.

Pseudorecombinant bipartite viruses produced by exchange of genomic DNA components of infectious clones of two strains of TGMV revealed that the determinant of symptom development in these viruses mapped to the common region and the *bc1* gene (von Arnim and Stanley, 1992). This conclusion was further substantiated when transgenic *N. benthamiana* plants expressing SLCV BC1 protein displayed symptoms that resembled those produced by the wild-type virus-infected plants (Pascal *et al.*, 1993). In contrast, transgenic plants expressing a truncated BC1 product or the full-length BV1 protein displayed normal plant phenotypes. It had been previously assumed that disease symptoms resulting from SLCV infection were due to virus-induced necrosis in the phloem companion cell that inhibited general transport throughout the phloem (Goodman, 1981). Further infectivity studies with mutants of SLCV with either deletions or introduced mutation in the BC1 and BV1 ORFs led to confirmation of the previous findings that the BC1 product was directly responsible for the pathogenic properties of SLCV (Ingham *et al.*, 1995). Likewise, many of the transgenic tobacco plants transformed with the *bc1* gene of ToMoV and BDMV also developed symptoms resembling virus-infection (Duan *et al.*, 1997; Hou *et al.*, 2000).

1.6 Phylogenetic relationships between geminiviruses

Phylogenetic analyses of known sequences of geminiviruses (Howarth and Vandemark, 1989; Padidam *et al.*, 1995a; Bradeen *et al.*, 1997) indicate that there are two major groupings corresponding to the mastreviruses and begomoviruses, with a minor group of curtoviruses. From an evolutionary point of view, an ancient monopartite virus may have been the progenitor of modern geminiviruses. The begomoviruses infecting dicots may have evolved later via genome duplication and reduction with partitioning of the replication and movement functions divided between both genomic components. Curtoviruses may have arisen from recombination of mastreviruses and begomoviruses and reduction to the monopartite form (Bradeen *et al.*, 1997).

The phylogenetic analyses also show that the whitefly-transmitted viruses form two clusters which have been named "New World" and "Old World" sub-divisions to reflect their geographic origins (Howarth and Vandemark, 1989). The viruses which cause leaf curl diseases of tomato were found to group with the "Old World" viruses, whereas the viruses that induced mosaic (TGMV) and mottle symptoms (ToMoV) in tomato, grouped with the "New World" viruses (Padidam *et al.*, 1995b). Viruses with sequence similarities <90% have been considered as distinct virus species whereas those species with >90% similarity are regarded as strains of the same virus (Padidam *et al.*, 1995b).

The analysis of coat protein sequences from a number of geminiviruses (Hong and Harrison, 1995), suggested that evolution of similar viruses in different regions has proceeded differently and they should be considered as separate species. Their dendrogram grouped 22 whitefly-transmitted geminiviruses into 3 distinct clusters,

representing viruses in the geographic regions of the Americas, the African-Mediterranean-Middle East, and the Asian-Australian region. Recently, a phylogenetic tree of DNA sequences from 64 geminiviruses including monopartite species and the DNA-A component of bipartite begomoviruses were aligned (Padidam *et al.*, 1999). The three widely accepted genera (*Mastrevirus, Curtovirus,* and *Begomovirus*) were supported in 1000 of 1000 bootstrapped trees (Figure 4).

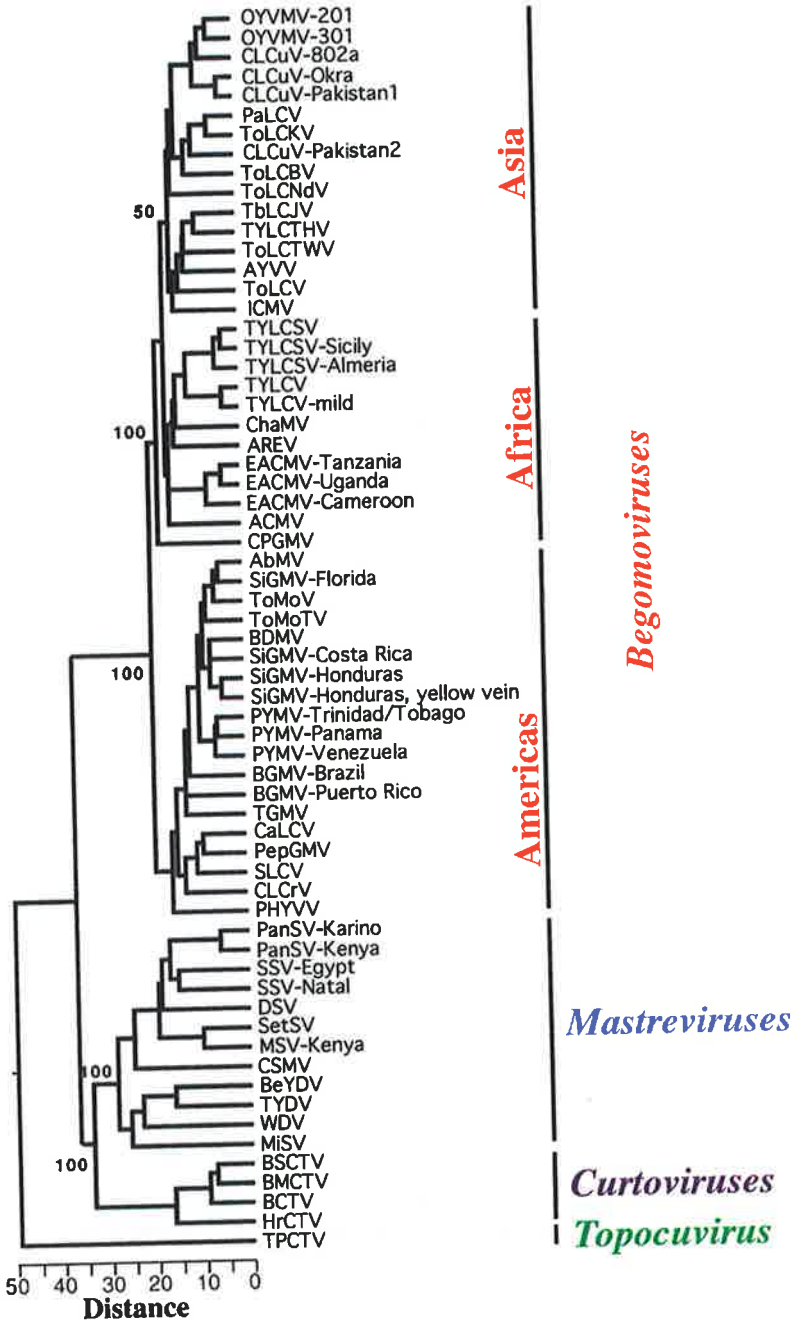


Figure 4. An unrooted phylogenetic tree of 64 geminivirus DNA sequences including viruses with a monopartite genome and the DNA-A component of bipartite begomoviruses (from Padidam et al. 1999). The tree was generated using the MegAlign program within DNASTAR software. Vertical distances are arbitrary and the scale shown indicates the distance between sequences. The bootstrap values for the regional clusters are indicated on the major branches.

1.7 Intramolecular and intermolecular recombination of geminiviruses

Homologous and non-homologous recombination events within the DNA of geminiviruses have been observed under experimental conditions (Brough *et al.*, 1988; Etessami *et al.*, 1988; Etessami *et al.*, 1989; Lazarowitz *et al.*, 1989; Hormuzdi and Bisaro, 1993; Bisaro, 1994). Genetic changes include reversion of deletion mutants to wild-type genome size, deletion of foreign sequences from geminivirus vectors, synthesis of a wild-type recombinant from two independent mutants, synthesis of subgenomic defective DNA molecules and the release of infectious viral DNA from recombinant plasmids containing monomeric genome inserts (Padidam *et al.*, 1999). The results of studies on experimental recombination indicated the possibility that some geminiviruses may have arisen naturally through DNA recombination events between unrelated co-replicating viruses.

Tests for recombination within the *Geminiviridae* (Padidam *et al.*, 1999), have identified 129 statistically significant recombination fragments distributed across various genomes (Figure 5 and 6). The lengths of the recombinant fragments varied from 32 to 2391 nucleotides. The begomoviruses from Asia appeared to have more examples of putative recombination with 49 possible recombinations between 16 viruses. Selected begomoviruses from Africa appeared to have 13 recombinations between 12 viruses whereas 36 recombinations were possible within 19 viruses from the Americas. Twelve examples of recombination were suspected within 5 curtoviruses and 15 potential recombinations were also possible among 12 mastreviruses. Some representative examples of recombinant fragments between pairs of viruses are shown in Table 5. No examples of recombination were detected between curtoviruses and mastreviruses.

Table 5. Examples of recombinant fragments identified within *Geminiviridae.*

Virus pair	Fragment (nts)	Start Virus 1	Start Virus 2	Probability
Mastrevirus				
PanSV-Kar & SSV-Egypt	92	720	704	7.2×10^{-5}
MSV-Ke & PanSV-Ke	88	273	293	3.3×10^{-4}
BeYDV & TYDV	106	1469	1502	7.3×10^{-3}
DSV & MSV-Set	47	257	277	1.4×10^{-2}
Curtovirus				
BCTV-Cal & TPCTV	176	2503	2368	5.9×10^{-28}
BCTV-Wor & HrCTV	236	705	625	1.1×10^{-9}
Curtovirus & Begomovirus				
BCTV-Cal & BGMV-PR	211	2479	2071	7×10^{-10}
HrCTV & SLCV	98	2593	2114	1.7×10^{-9}
Topocuvirus & Begomovirus				
TPCTV & ToMoV	359	2372	2061	1.2×10^{-22}
TPCTV & ICMV	186	2434	2359	1.6×10^{-16}
Begomovirus- Asia				
CLCuV-802a & OYVMV-201	1494	2732	2727	7.3×10^{-33}
ToLCNdV & ToLCBV	933	686	689	9.7×10^{-18}
TbLCV & TYLCTHV	1195	1190	1191	2.5×10^{-11}
AYVV & ICMV	125	2286	2366	1.5×10^{-3}
Begomovirus- Africa				
EACMV-CM & EACMV-Tz	1949	1973	1972	1.7×10^{-17}
TYLCV & TYLCV-m	67	67		7.9×10^{-15}
TYLCSV & TYLCSV-Sic	2241	143	143	6.9×10^{-6}
ChaMV & TYLCV	224	254	224	1.9×10^{-3}
Begomovirus- Americas				
BDMV & PHYVV	326	1998	2023	6.1×10^{-13}
PYMV-Ven & ToLCV-Panama	1625	133	134	2.9×10^{-8}
CLCrV & PYMV-TT	690	1025	997	5.9×10^{-4}
SiGMV-Hn & SiGMV-Yv	556	941	949	3.7×10^{-3}
Begomovirus- Asia /Africa				
AREV & ToLCV	316	2098	2097	1.6×10^{-7}
AREV & OYVMV-301	299	298	2080	5×10^{-13}
ICMV & TYLCSV	104	2366	2292	1.45×10^{-5}
ToLCNdV & TYLCV	200	2194	2213	4.2×10^{-4}

Data extracted from Padidam *et al.*, 1999.

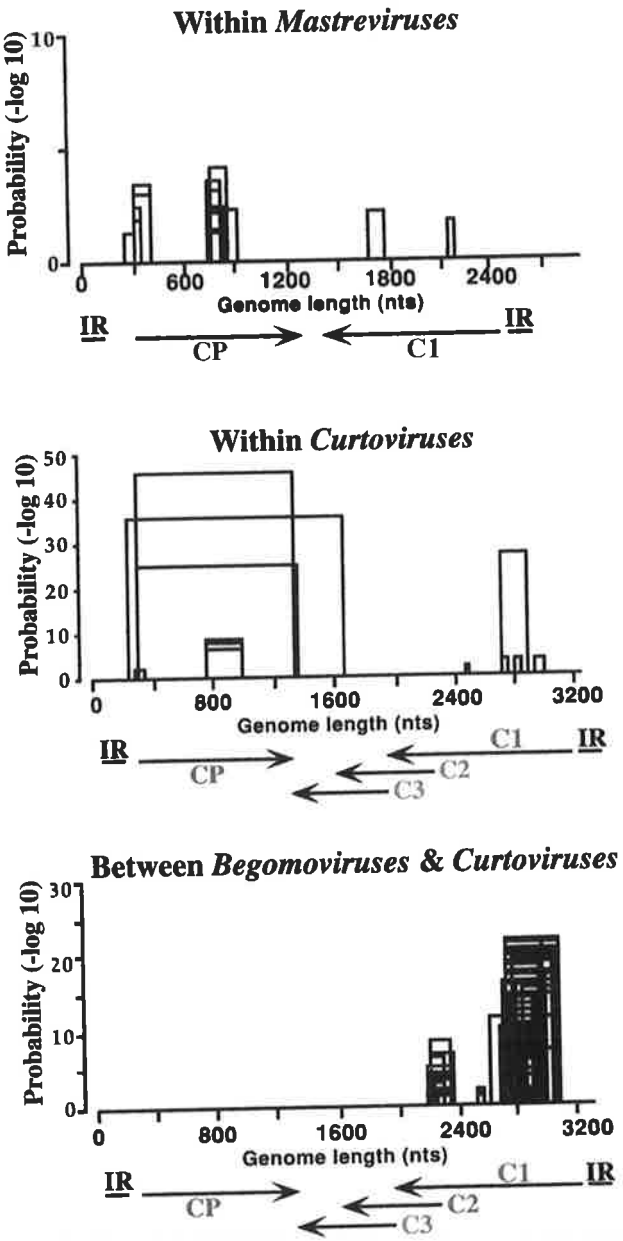


Figure 5. Prediction of recombination events within the genome of mastreviruses, curtoviruses and between begomoviruses and curtoviruses (from Padidam *et al.*, 1999). The beginning and the end of the sequence that potentially underwent recombination is connected by a line to form an open rectangle. The height of the open rectangle is proportional to $-\log_{10}$ of the probability (P) of recombination. Only values of P that are <0.05 are shown. Relative positions of the ORFs and the intergenic region (IR) are indicated.

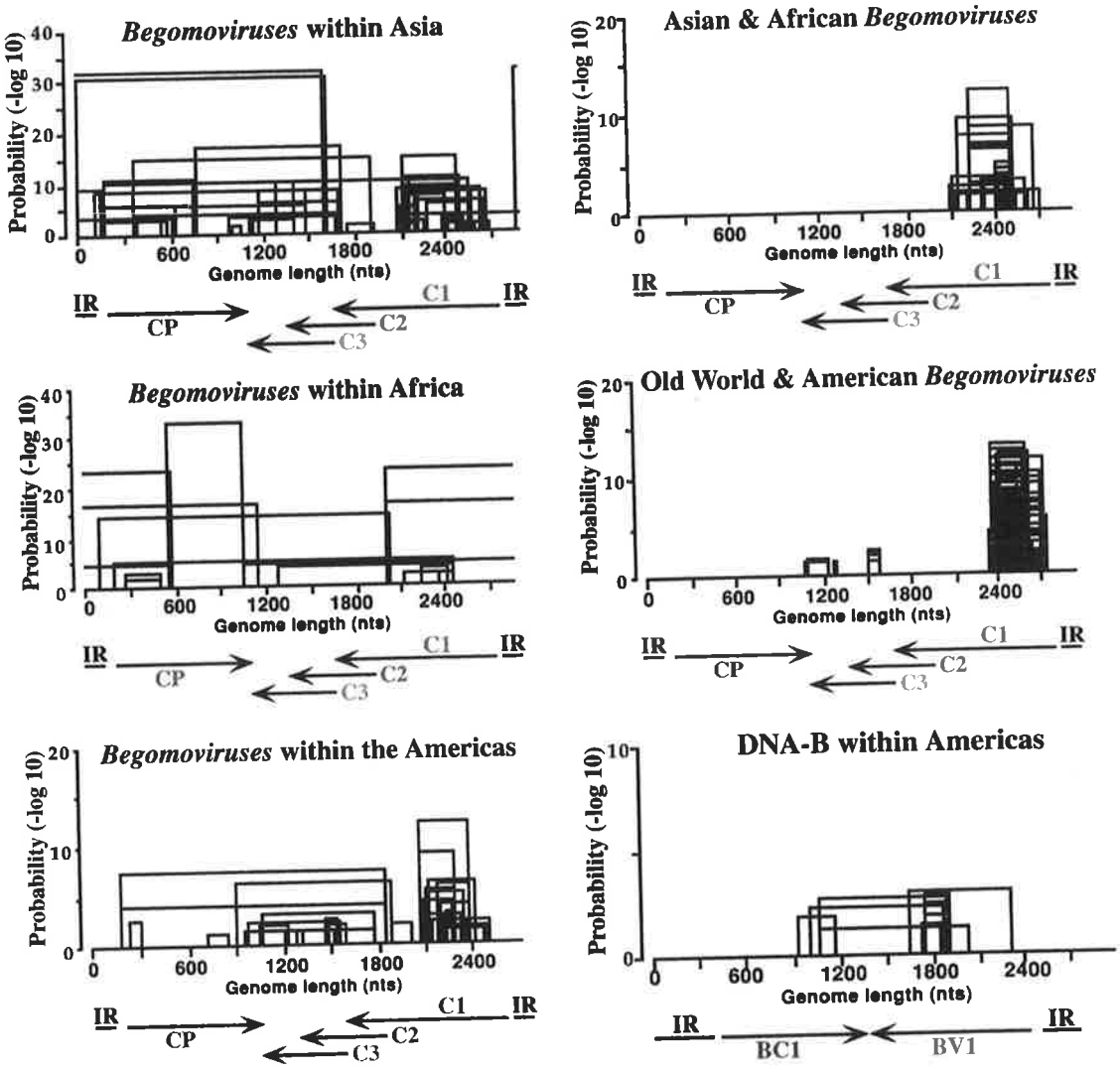


Figure 6. Prediction of recombination events within the genome of begomoviruses in different regions of the world (extracted from Padidam et al. 1999). The beginning and the end of the sequence that potentially underwent recombination is connected by a line to form an open rectangle. The height of the open rectangle is proportional to $-\log_{10}$ of the probability of recombination. Only P values that are < 0.05 are shown. Relative positions of the ORFs and the intergenic region (IR) are indicated.

1.8 Tomato diseases caused by begomoviruses

Begomoviruses have caused diseases in tomato crops in both tropical and subtropical regions (Bird and Maramorosch, 1978; Makkouk and Latterrot, 1983; Czosnek *et al.*, 1990; Cohen and Antignus, 1994; Green and Kalloo, 1994). These viral diseases were first identified by foliar symptoms and host range before the causal viruses were isolated and characterised. More than 30 distinct begomoviruses are recognised as pathogens of the tomato plant. One or more of these viruses have caused tomato diseases in at least 50 different countries. The effect of begomoviruses on regional tomato production varies from year to year with the flux of disease epidemics.

Tomato plants infected with begomoviruses are usually stunted with smaller than normal leaflets and display deformities including rugosity, puckering, and curling of leaf margins. The laminae may have a golden or a light yellow mosaic or pale green mottled appearance or alternatively, only the leaf margins may be abnormally yellow or chlorotic. Depending on the stage of plant development when the virus infection occurs, the virus-infected tomato plants may survive to flower, but produce either little or no fruit because of continual flower drop (Green and Kalloo, 1994).

1.8.1 Transmission and spread

There are no reported cases of transmission of begomoviruses via seed. Although some geminiviruses are mechanically transmissible, the epidemiology of transmitted virus disease is characterised by a close correlation between disease incidence and insect vector populations. This relationship is obvious even with seasonal fluctuations (Green and Kalloo, 1994). The majority of whitefly-mediated diseases of tomatoes are caused by begomoviruses and transmitted by biotypes of *Bemisia tabaci* (Gennadius) (Cohen

and Harpaz, 1964; Cohen and Nitzany, 1966; Costa, 1969; Costa, 1976; Cohen and Antignus, 1994; Caciagli *et al.*, 1995). These insects are commonly known as tobacco, sweet potato, or cotton whiteflies and also as the biotype-A whitefly. During the late 1980's, a new biotype-B whitefly (also known as the poinsettia or silverleaf whitefly or *B. argentifolii* (Bellows & Perring)) was identified in the USA (Costa and Brown, 1991; Bellows *et al.*, 1994). Subsequently, these biotype-B whiteflies have been found in the Middle East, Mediterranean region, Caribbean Islands, Central and North America, Africa, Japan, southern Europe, glasshouses of northern Europe and in Australia (Costa *et al.*, 1993; Bedford *et al.*, 1994a; Brown, 1994; Markham *et al.*, 1994; Gunning *et al.*, 1997).

Under favourable conditions, the biotype-B whiteflies are capable of producing 11-15 generations in one year (De Barro, 1995). In some areas where they have become established, they have displaced the native biotype-A whitefly population (Bird and Brown, 1998; Toscano *et al.*, 1998). Each biotype-B female can produce between 100-300 eggs, giving rise to very large populations of insects that are capable of damaging the plants directly by feeding causing silverleaf of squash and irregular ripening of tomato. As the host range of the B-biotype exceeds 500 plant species including economically important crops, these insects are capable of transmitting begomoviruses into new hosts.

Currently, more than 50 different begomoviruses have been recorded as being transmitted by *B. tabaci* to plants in different regions of the world (Briddon and Markham, 1995). Transmission of begomoviruses by *B. tabaci* to tomato is widely accepted to be persistent and circulative (Cohen and Harpaz, 1964; Cohen and Nitzany,

1966; Costa, 1976; Bird and Maramorosch, 1978; Duffus, 1987; Brown, 1994; Caciagli *et al.*, 1995; De Barro, 1995; Rubinstein and Czosnek, 1997). The efficiency of viral transmission appears to be influenced by both the viral isolate and vector biotype (McGrath and Harrison, 1995). Although the mechanism is not understood, it is believed that the capsid protein is involved (Harrison, 1985; Briddon *et al.*, 1990; Brown, 1994).

It is also generally recognised that female whiteflies are more efficient viral vectors than males, possibly due to differences in feeding habit (Costa, 1969). In general terms, *B. tabaci* can aquire virus from infected plants during a feeding period as short as 10 minutes, but the efficiency of transmission increases with feeding periods up to 24 hours. Usually transmission occurs after a latent period of 4-21 hours. A viruliferous whitefly can transmit the virus for at least 5-20 days with a gradual loss of transmission efficiency over time. The virus passes through moults from nymphs to adults (Cohen and Nitzany, 1966; Butler and Rataul, 1977; Bird and Maramorosch, 1978; Brown, 1991; Cohen and Antignus, 1994; Green and Kalloo, 1994; De Barro, 1995).

Whiteflies carrying the Israeli isolate of *Tomato yellow leaf curl virus* (TYLCV) have been shown to exhibit periodic acquisition (Cohen and Harpaz, 1964). This term has been used to define the events post-aquisition, when viruliferous insects losing infectivity were prevented from aquiring more viral inoculum from infected plants. Further aquisition of inoculum was only restored following the complete loss of initial infectivity. This phenomenon was also observed with ToYMV in India (Verma *et al.*, 1975), although not unequivocally proved for TYLCSV (Caciagli *et al.*, 1995). An antiviral factor was detected in homogenates of viruliferous whiteflies, and the feeding

of this factor to virus-free whiteflies prior to or after virus acquisition, reduced their ability to transmit the virus (Cohen and Marco, 1970). The DNA of TYLCV has been detected in whiteflies by dot-blot hybridisation as early as 1 hour after acquisition access. The increase in viral DNA accumulation was correlated to the length of acquisition access between 1-96 hours (Antignus *et al.*, 1994). The retention of the viral DNA in the vector was at least 23 days, but the viral coat protein could not be detected beyond 10 days after the acquisition access. Further, no TYLCV replication was observed in viruliferous whiteflies after their feeding on a ^{32}P-labelled nucleotide. It was concluded that whiteflies could not accumulate naked viral single-stranded DNA, cloned viral double-stranded DNA or virions with impaired coat protein.

More recent studies showed that TYLCV DNA remained associated with a viruliferous whitefly for its entire adult life and that the viral DNA was passed through the egg on to the progeny instars, then adults for at least two generations (Rubinstein and Czosnek, 1997; Ghanim *et al.*, 1998). The adult progeny of the second generation from viruliferous insects were able to infect tomato test plants indicating that the viruliferous whiteflies and their progeny, should be able to transmit TYLCV from crop to crop in the absence of alternative reservoir plant hosts. Further studies under controlled conditions revealed that viruliferous whiteflies could pass on TYLCV to other insects in the absence of any other source of virus indicating that sexual transmission of the virus from insect to insect had occurred (Ghanim and Czosnek, 2000). The transmission of virus from insect-to-insect would quickly increase the build-up of viruliferous insects during periods of pest epidemics.

1.8.2 Effect of begomoviruses on regional tomato production

In the year 2000, total tomato production throughout the World was estimated to be near to 99 million tonnes (FaoStat Database-http://apps.fao.org), despite the on-slaught of the many and various pests and diseases including viruses that parasitise the tomato plant. The average yearly production per hectare of tomatoes estimated in Africa, Asia, North, Central America, South America and Europe during the period from 1961 to 2000 is shown in Figure 7. The relevance of these regional statistics and sub-regional tomato production (Figures 8-15) in relation to reported crop loss of tomatoes due to geminivirus infection is discussed in Chapter 5.

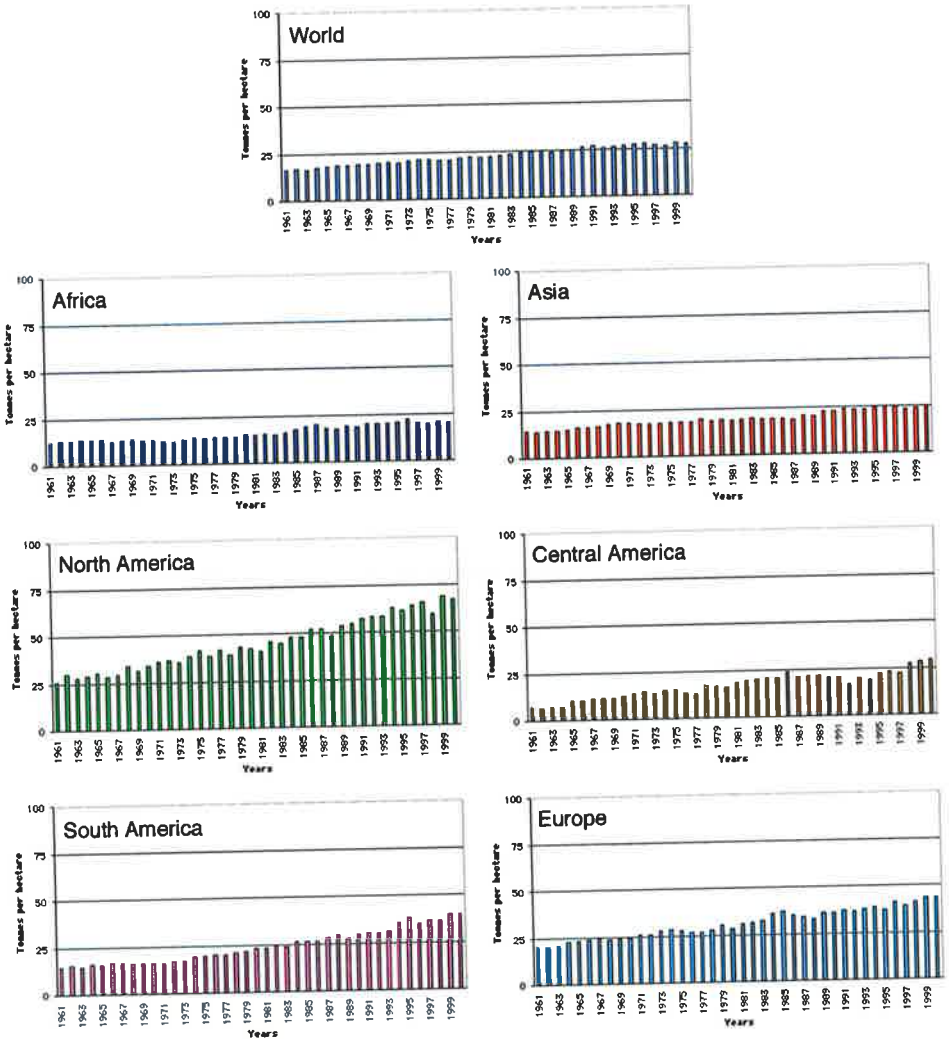


Figure 7. World and regional trends in average yearly tomato production per hectare during the period from 1961 to 2000 (Source: FaoStat)

1.8.2.1 Middle East, Africa and Mediterranean region

Whiteflies were first associated with an epidemic of tomato leaf curl disease in the Middle East during 1939-40 (Cohen and Antignus, 1994). Some twenty years later, following another outbreak of *B. tabaci* in the Jordan and Bet She'an Valley, some of the tomato crops were completely destroyed by TYLCV (Cohen and Harpaz, 1964). Reports indicated that outbreaks of TYLCV in Israel were only sporadic during the 1960's (Cohen and Harpaz, 1964), but in recent decades this virus disease has become a serious economic problem (Czosnek and Laterrot, 1997). Occurrences of TYLCV have been found in other areas of tomato production within the Middle East (Table 6).

Table 6. First reports of tomato yellow leaf curl disease in the Middle East

Country	Disease identified	Virus identified	Reference
Israel	1939	1988	(Czosnek *et al.*, 1990; Cohen and Antignus, 1994)
Egypt	1966	1989	(Czosnek *et al.*, 1990; Nakhla *et al.*, 1992; Mazyad *et al.*, 1994; Aref and El-Dougdoug, 1996)
Saudi Arabia	1971		(Mazyad *et al.*, 1979)
Jordan	1976	1994	(Makkouk, 1978; Al Musa, 1982; Grit *et al.*, 1994)
Lebanon	1976	1989	(Makkouk, 1976; Czosnek *et al.*, 1990)
Iraq	1978		(Makkouk, 1978)
Turkey	1988	1988	(Navot *et al.*, 1989)
Yemen	1990	1990	(Bedford *et al.*, 1994b)
Azerbaidjan, Turkmenastan, Uzbekistan	1993		(Czosnek and Laterrot, 1997)
Oman	1994		(Zouba, 1995)
Iran	1995	1995	(Hajimorad *et al.*, 1996)

The yearly variation of average tomato production per hectare from 1961 to 2000 for six representative Middle East countries where TYLCV occurs is shown in Figure 8.

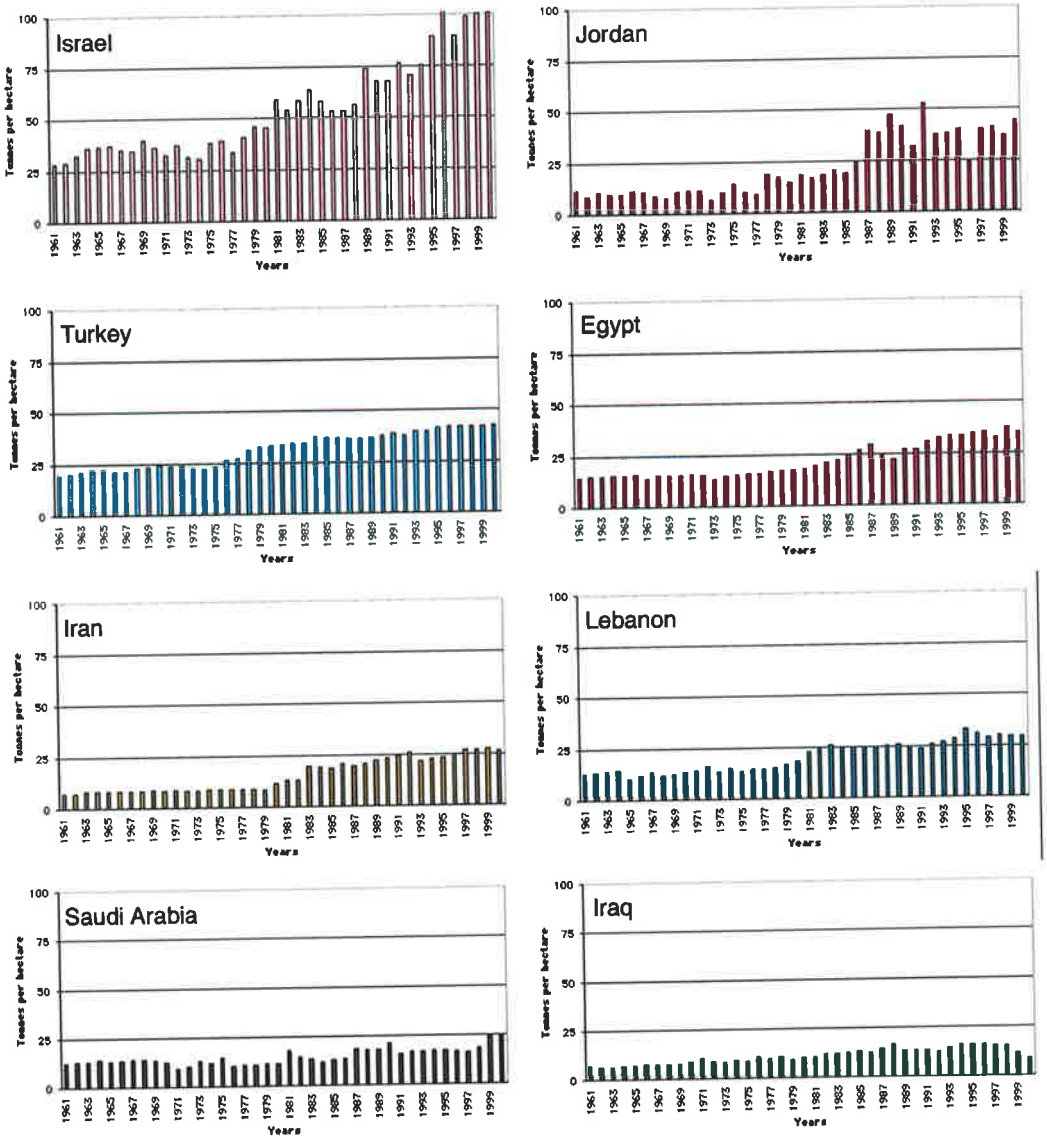


Figure 8. Average yearly tomato production (tonnes per hectare) between 1961 and 2000 for Israel, Jordan, Turkey, Egypt, Iran, and Lebanon (Source: FaoStat).

Examples of reported incidence of tomato yellow leaf curl disease and estimated crop loss of tomatoes are shown in Table 7. The higher disease incidence occurred during the summer and autumn seasons (Yassin and Nour, 1965; Nitzany, 1975; Makkouk *et al.*, 1979; Al Musa, 1982; Makkouk and Latterrot, 1983; Ioannou, 1985b; Pico *et al.*, 1996). The regional hosts for whiteflies and alternative hosts for TYLCV are shown in Table 8.

Reports of the incidence of TYLCV in the African and Mediterranean region (Table 9) and their effect on tomato production is also shown in Table 7. The yearly tomato production of representative countries in this region is shown in Figure 9. Leaf curl disease of tomato has been known in Tunisia since 1967 and a similar disease was first observed in Cyprus during 1974 (Cherif and Russo, 1983; Ioannou, 1985). During 1988 and 1989, the first outbreaks of tomato yellow leaf curl disease were observed in whitefly-infested glasshouse-grown tomatoes in Sardinia and Sicily (Credi *et al.*, 1989; Luisoni *et al.*, 1989). Both TYLCV and *B. tabaci* are now endemic in these regions.

Epidemic outbreaks of tomato yellow leaf curl disease first occurred in southern Spain at Murcia and Almería during 1992 (Moriones *et al.*, 1993). Since then, the disease has spread to all of the major regions of vegetable production in southern and south-eastern Spain. Isolates of TYLCSV were found in the indigenous *Solanum nigrum* weeds and associated with disease epidemics (Bedford *et al.*, 1998). During 1997, an abnormally severe disease outbreak occurred in the Almerían tomato crops which was caused by TYLCV (Navas-Castillo *et al.*, 1997; Navas-Castillo *et al.*, 1999).

Table 7. The incidence and estimated crop loss due to tomato yellow leaf curl disease.

Country	Season	Incidence	% Loss	Reference
Israel, Jordan	Summer Greenhouse Spring	93-100% 0-14%	50-75% 63%	(Nitzany, 1975; Makkouk, 1978; Al Musa, 1982)
Lebanon	Summer Greenhouse	85-90% 28-50%	63%	(Makkouk *et al.*, 1979; Abou Jawdah *et al.*, 1995)
Egypt	Autumn	100%	80-99%	(Mazyad *et al.*, 1979; Nakhla *et al.*, 1993)
Saudi Arabia	Autumn Winter	58-100% 4-6%	30-80%	(Mazyad *et al.*, 1979)
Cyprus	Summer Winter	20-100% nil	50-82%	(Ioannou, 1985b)
Tunisia	Autumn	20-100%		(Cherif and Russo, 1983)
Sardinia Sicily	Greenhouse	80%		(Credi *et al.*, 1989; Gallitelli *et al.*, 1991)
Calabria	Spring	6%		(Polizzi and Areddia, 1992)
Spain	Autumn, Winter	70%		(Moriones *et al.*, 1993)

Table 8. Known alternative hosts for whiteflies and begomoviruses affecting tomato.

Country	Virus	Host plants for *B. tabaci*	Plant hosts for begomoviruses
Israel, Jordan	TYLCV	*Cucumis sativus, Solanum melongena, Capsicum annum, Ipmoea batatas, I. purpurea, Lantana camarana, Lantana montevidensis, Melissa officinalis, Alhagi graecorum, Lippia nodiflora, Weddelia palludosa, Gaillardia pulchella,*	*Nicotiana tabacum, Phaseolus vulgaris, Lens esculenta, Datura stramonium, Malva nicaensis, Malva parviflora, Cynanchum acutum*
Italy	TYLCV	*Solanum melongena, S. tuberosum, Capsicum annum, N. tabacum, Gossypium hirsutum, Helianthus annum, Capparis sp., Chenopodium sp., Convolvulus sp., Datura sp., Euphorbia sp., Malva sp., Sonchus sp., Teucrium sp.*	*Solanum nigrum, Datura stramonium, Euphorbia sp.*
Spain	TYLCV	Cucurbit crops, *Solanum nigrum*	*Solanum nigrum, S. luteum, D. stramonium, Mercurialis ambigua*
Cyprus	TYLCV	*N. tabacum. D. stramonium*	*N. tabacum. D. stramonium*
Turkey	TYLCV	*D. stramonium, S. nigrum, Urtica* sp.	*D. stramonium, S. nigrum, Urtica* sp.

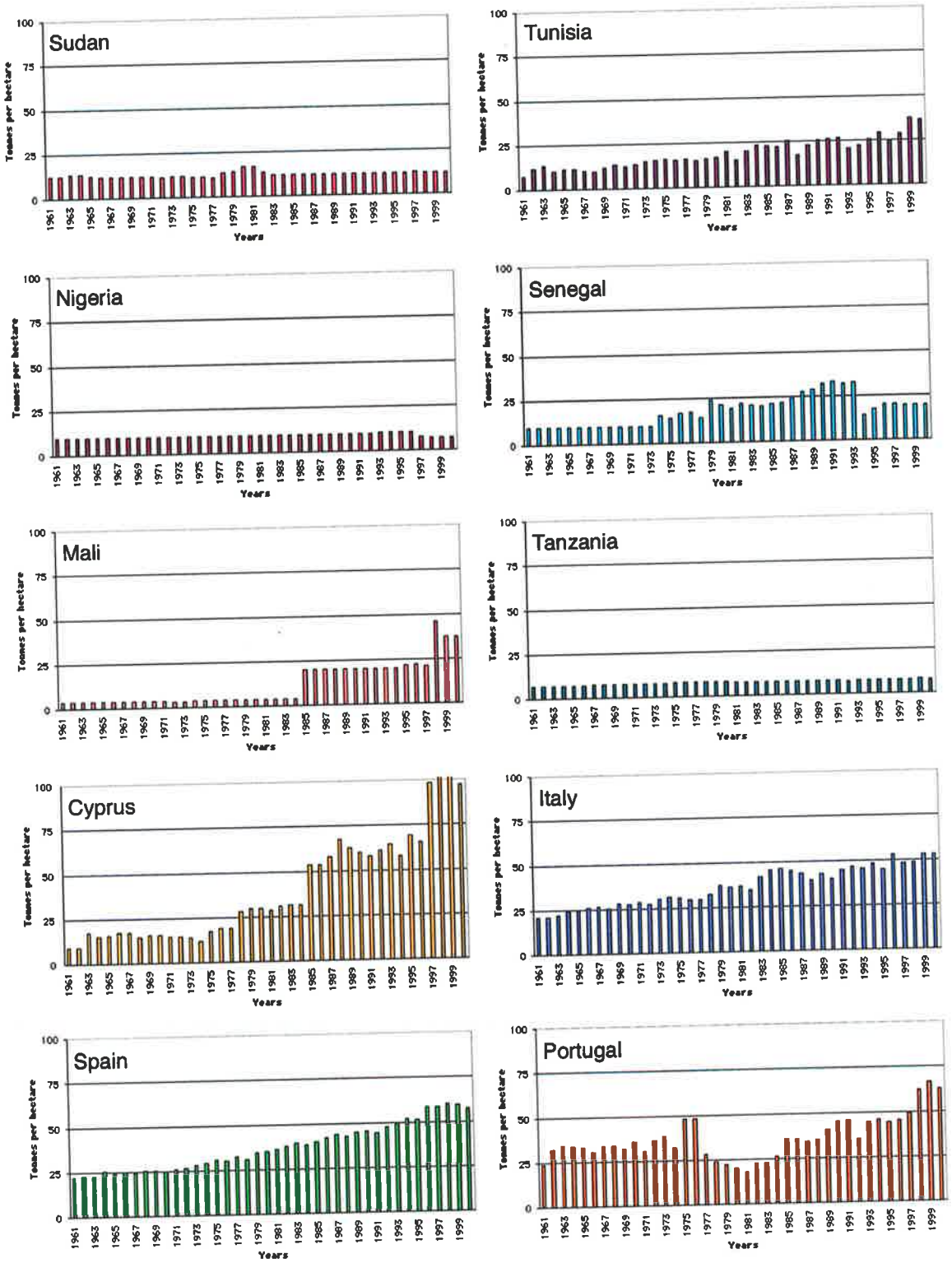


Figure 9. Examples of yearly tomato production (tonnes per hectare) between 1961 and 2000 in the African and Mediterranean region. (Source: FaoStat).

TYLCV has rapidly spread throughout southern Spain and has progressively displaced strains of TYLCSV. The reason for this may be due partly to the ability of TYLCV to infect the common bean (*Phaseolus vulgaris* L.) that is grown between tomato crops and also because it is more efficiently spread by biotypes of *B. tabaci* in this locality (Sanchez-Campos *et al.*, 1999)

Table 9. First reports of tomato yellow leaf curl disease in Africa and the Mediterranean.

Country	Disease identified	Virus identified	Reference
Sudan	1965		(Yassin and Nour, 1965; Kisha, 1981)
Tunisia	1967		(Cherif and Russo, 1983)
Cyprus	1974	1989	(Ioannou, 1985; Czosnek *et al.*, 1990)
Nigeria	1976	1988	(D'Hondt and Russo, 1985; Czosnek *et al.*, 1990)
Senegal	1976	1987	(D'Hondt and Russo, 1985; Czosnek *et al.*, 1990)
Gambia	1978		(D'Hondt and Russo, 1985)
Mauritania	1979		(D'Hondt and Russo, 1985)
Ivory Coast	1980		(D'Hondt and Russo, 1985)
Mali	1983	1988	(D'Hondt and Russo, 1985; Czosnek *et al.*, 1990)
Sardinia	1988	1988	(Luisoni *et al.*, 1989; Czosnek *et al.*, 1990; Gallitelli *et al.*, 1991; Kheyr-Pour *et al.*, 1991)
Sicily	1989	1990	(Credi *et al.*, 1989; Czosnek *et al.*, 1990)
Calabria	1991		(Polizzi and Areddia, 1992)
Spain	1992		(Moriones *et al.*, 1993; Noris *et al.*, 1994; Navas-Castillo *et al.*, 1997; Navas-Castillo *et al.*, 1999)
Tanzania	1994		(Chiang *et al.*, 1997)
Portugal	1995	1995	(Louro *et al.*, 1996)
Burkina Faso	1995	1995	(Konate *et al.*, 1995)

1.8.2.2 Asia

Several reports indicate that tomato leaf curl diseases are widespread in Asia and caused by different begomoviruses (Table 10). In India, a tomato disease characterised by yellowing and leaf curling was recorded in 1946 (Vasudeva and Samraj, 1948). An epidemic of tomato leaf curl disease was again observed during the 1963-64 season (Muniyappa *et al.*, 1984), and a yellow mosaic disease was found during 1974 (Verma *et al.*, 1975). The known alternative hosts for tomato leaf curl virus in the Indian region include *Euphorbia* sp., *Acanthospermum* sp., *Parthenium* sp., and *Ageratum* sp.

Table 10. First reports of leaf curl diseases of tomato in Asia

Country	Disease identified	Virus identified	Reference
India	1946	1994	(Vasudeva and Samraj, 1948; Grit *et al.*, 1994)
Taiwan	1981	1988	(Green *et al.*, 1987; Czosnek *et al.*, 1990)
Thailand	1983	1988	(Thanapas *et al.*, 1983; Czosnek *et al.*, 1990; Rochester *et al.*, 1990)
Nepal	1990		(Dahal *et al.*, 1993)
China	1998	1998	(Liu *et al.*, 1998)

Tomato leaf curl diseases have also been found in Nepal (Dahal *et al.*, 1993), Pakistan (Harrison *et al.*, 1997; Mansoor *et al.*, 1997), China (Liu *et al.*, 1998), Taiwan (Green *et al.*, 1987), Thailand (Thanapas *et al.*, 1983), Cambodia, Indonesia, Malaysia, and the Philippines (Green and Kalloo, 1994). Recently, a variant of the Israeli isolate of TYLCV that induces mild symptoms in tomato has been isolated from affected tomato plants in Japan (Kato *et al.*, 1998). The yearly variation in average tomato production per hectare for India, Pakistan, China and Thailand during the period between 1961 to 2000 is shown in Figure 10.

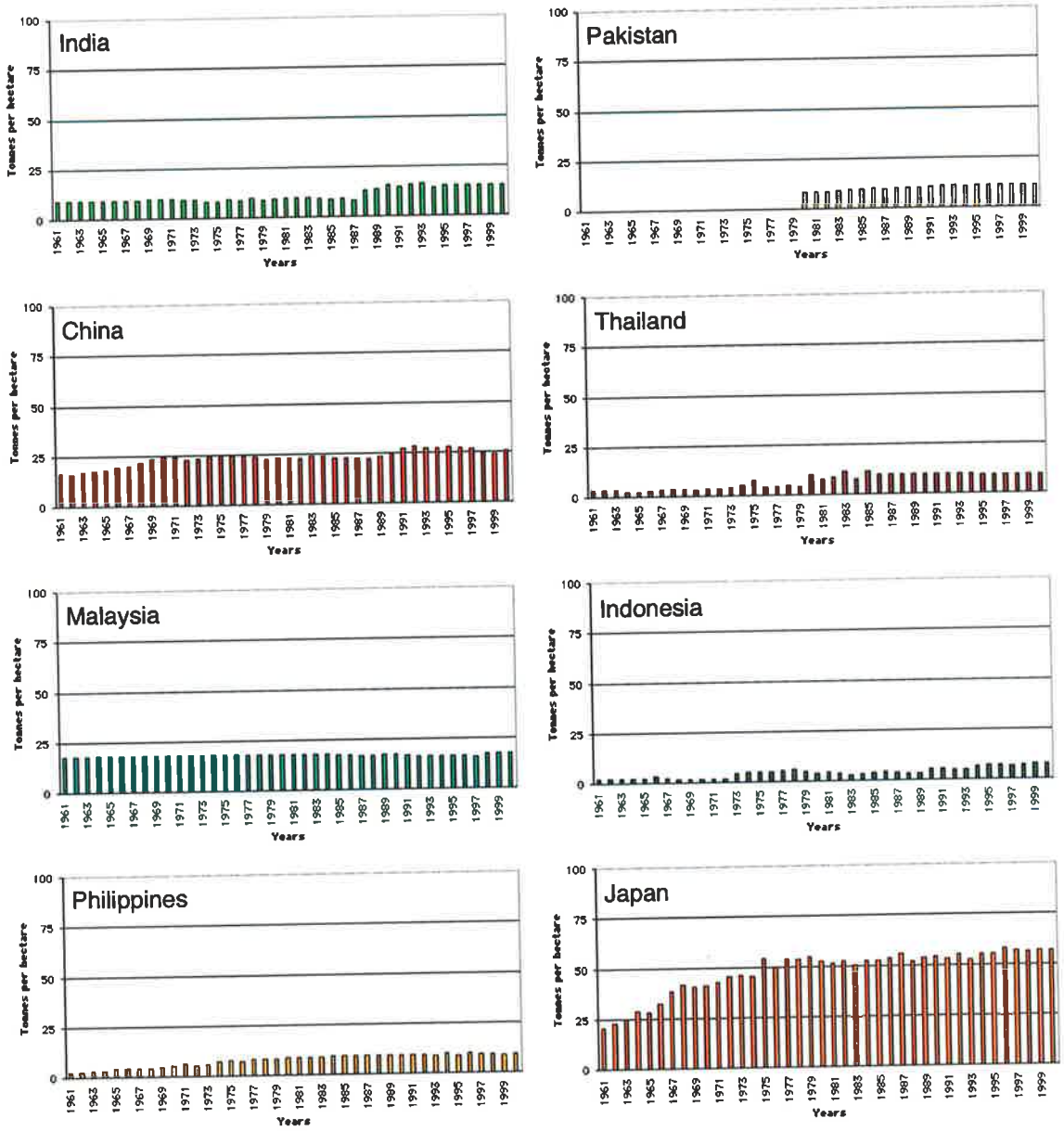


Figure 10. Average yearly tomato production (tonnes per hectare) between 1961 and 2000 for India, Pakistan, China and Thailand (Source: FaoStat).

1.8.2.3 The Americas

Whitefly-transmitted geminiviruses have caused loss of tomato production in South America since the late 1960's (Brown, 1991; Brown and Bird, 1992; Brown *et al.*, 1995; Polston and Anderson, 1997). Previously, geminiviruses and *B. tabaci* co-existed for many decades without affecting cultivated crops and their emergence as economic problems are the result of drastic changes in traditional cropping systems (Morales and Anderson, 2001). Currently, five million hectares of different crops in twenty countries are under attack from more than 30 different begomoviruses. The variation in yearly average tomato production for several countries that produce tomatoes is shown in Figure 11.

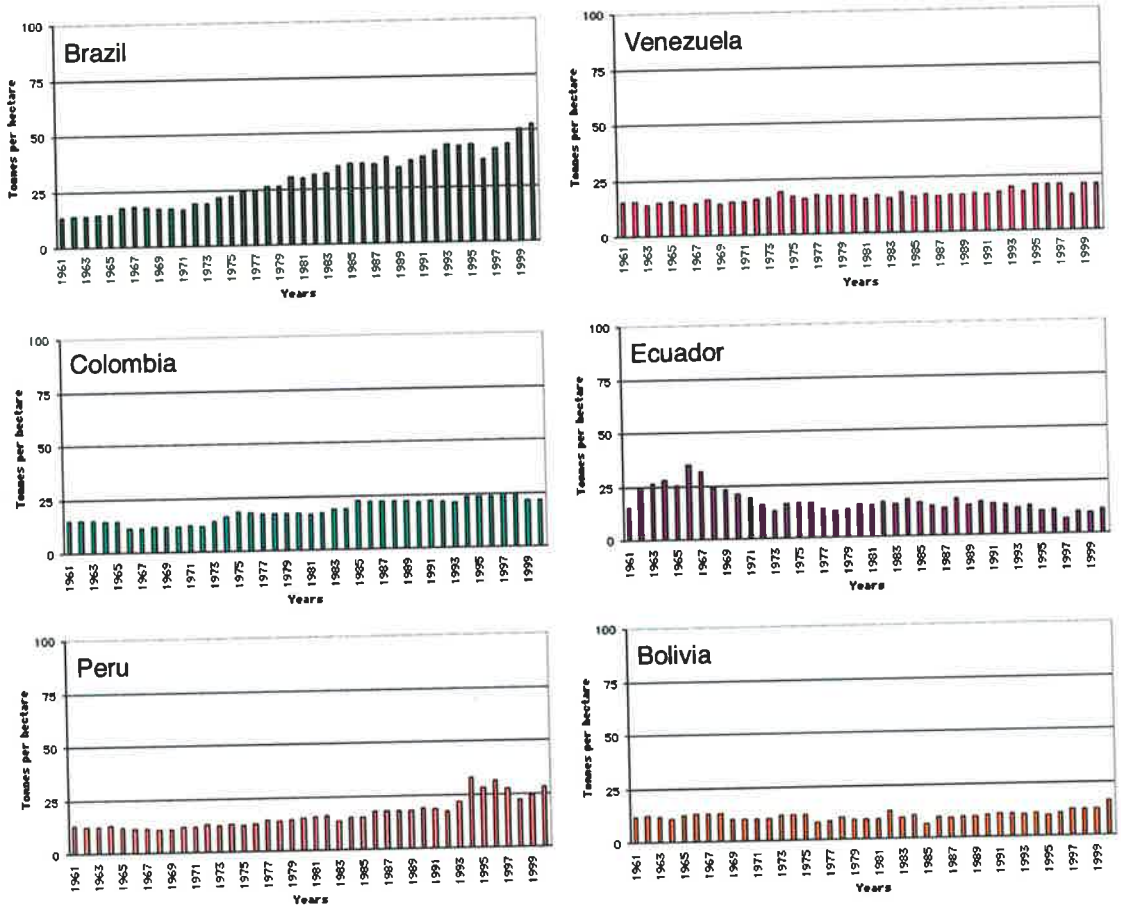


Figure 11. Average yearly tomato production (tonnes per hectare) between 1961 and 2000 for several representative countries in South America (Source: FaoStat).

Infectious chlorosis of tomato or tomato golden mosaic was reported in Brazil during the 1960's (Flores *et al.*, 1960; Costa, 1969). The causal agent was subsequently identified as TGMV (Matyis *et al.*, 1975). Since 1994, the invasion of tomato crops by the B-biotype of *B. tabaci* has led to increased incidence of tomato virus symptoms in Brazil (Ribeiro *et al.*, 1998). In some cases, the incidence of virus symptoms was reported to be close to 100% and no tomato fruit of commercial value could be harvested from the affected fields (Ribeiro *et al.*, 1998). Although TGMV has not been detected in recent tomato surveys in Brazil, other geminiviruses have emerged as important viruses (Polston and Anderson, 1997). In the state of Minas Gerais, viruses provisionally named TGV-BZ-Ig and TGV-BZ-Ub were isolated from plants exhibiting yellow mosaic symptoms. Another virus was isolated in the state of São Paulo and named *Tomato yellow vein streak virus* (ToYVSV) (Faria *et al.*, 1997). Further geminivirus species have been found in the states of Rio de Janeiro, Bahia, Pernambuco and the Federal District (Polston and Anderson, 1997).

An epidemic of "mosaico amarillo del tomate" or tomato yellow mosaic disease in Venezuela during 1961 forced many growers to eliminate their existing tomato plantings and to replant with new seedlings (Debrot and Dao, 1963; Debrot and Centeno, 1985). Throughout the 1960's and 1970's, tomato yellow mosaic disease was found to be a major limiting factor for tomato production in the states of Aragua, Carabobo, Guarico, and Lara. By the mid-1970's, nearly 100% of tomato plants surveyed at flowering showed characteristic symptoms of virus infection and ToYMV was identified as the common causal agent (Lastra and Uzcátegui, 1975). Irregular ripening of tomato fruit and infestation of tomato plants with *B. tabaci* B biotype whiteflies were first found in Venezuela during the 1990-1991 season (Polston and

Anderson, 1997). Subsequently, a strain of PYMV was identified from affected tomato plants in 1997 (Guzman *et al.*, 1997). Recently, it has been suggested that PYMV may be the same virus as ToYMV (Morales and Anderson, 2001).

Following outbreaks of high populations of *B. tabaci* biotype-B whiteflies in the Dominican Republic in 1987, several indigenous geminivirus species spontaneously affected tomato production in the south (Azua) and northwestern areas (Brown and Bird, 1992; Polston and Anderson, 1997). Some of the viruses found were related to PYMV and similar viruses also infect tomato plants in Trinidad and Tobago and several other locations in the eastern Caribbean (Polston and Anderson, 1997). In recent years, the viruses may have been disseminated through the movement of infected tomato transplants or through infected potato tubers or via the movement of plants harbouring viruliferous whiteflies or dispersal of viruliferous whiteflies by seasonal hurricanes (Polston and Anderson, 1997). The variation in yearly average tomato production for tomato producing regions of the Caribbean is shown in Figure 12.

In 1994, the Israeli isolate of TYLCV was unknowingly introduced into the Dominican Republic by a tomato grower via imported infected transplants of a fresh-market tomato variety from Israel (Nakhla *et al.*, 1994; Polston *et al.*, 1994; Polston and Anderson, 1997). Subsequently, TYLCV has been found widely in home gardens and in weed species and also the virus is now established in the agroecosystem of Cuba and Jamaica (McGlashan *et al.*, 1994; González and Valdés, 1995; Ramos *et al.*, 1996; Martinez-Zubiaur *et al.*, 1996; Martinez-Zubiaur *et al.*, 1997; Polston and Anderson, 1997). It is not known if all occurrences of TYLCV are the result of regional spread

from a single importation or due to dispersion from several different introductions of infected plants. Alternative hosts for TYLCV in the Caribbean region include *Boerhavia erecta, Cynanchum acutum,* and *D. stramonium.*

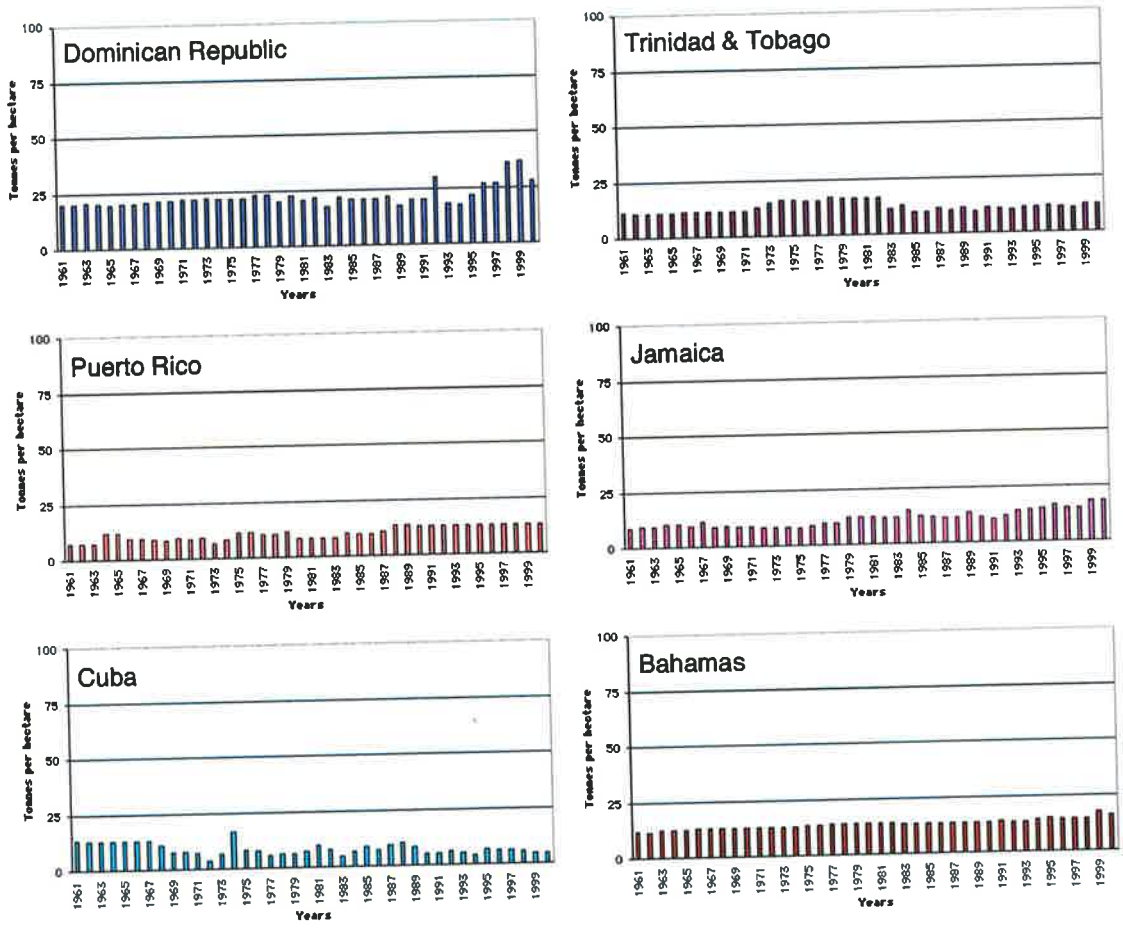


Figure 12. Average yearly tomato production (tonnes per hectare) between 1961 and 2000 for tomato producing regions of the Caribbean (Source: FaoStat).

The variation in yearly average tomato production for tomato producing regions of Central America is shown in Figure 13. In this area, whitefly-transmitted tomato diseases were reported to occur in most of the countries. In Nicaragua, significant outbreaks of whitefly-transmitted geminiviruses occurred in the Sebaco Valley of Nicaragua during 1983 and 1984 (Rosset, 1986). Since then, this problem has quickly

spread to all other tomato growing regions of the country and tomato production has been drastically reduced (Rojas *et al.*, 2000). Similarly, economically significant viral diseases of tomato were also found associated with *B. tabaci* in Guatemala during 1987, El Salvador and Costa Rica in 1988, Honduras in 1989, and Panama in 1991 (Polston and Anderson, 1997). Other virus disease epidemics of tomato occurred in Guadeloupe, Martinique and Puerto Rico during 1992, 1993, and 1994, respectively (Brown *et al.*, 1995; Polston *et al.*, 1998).

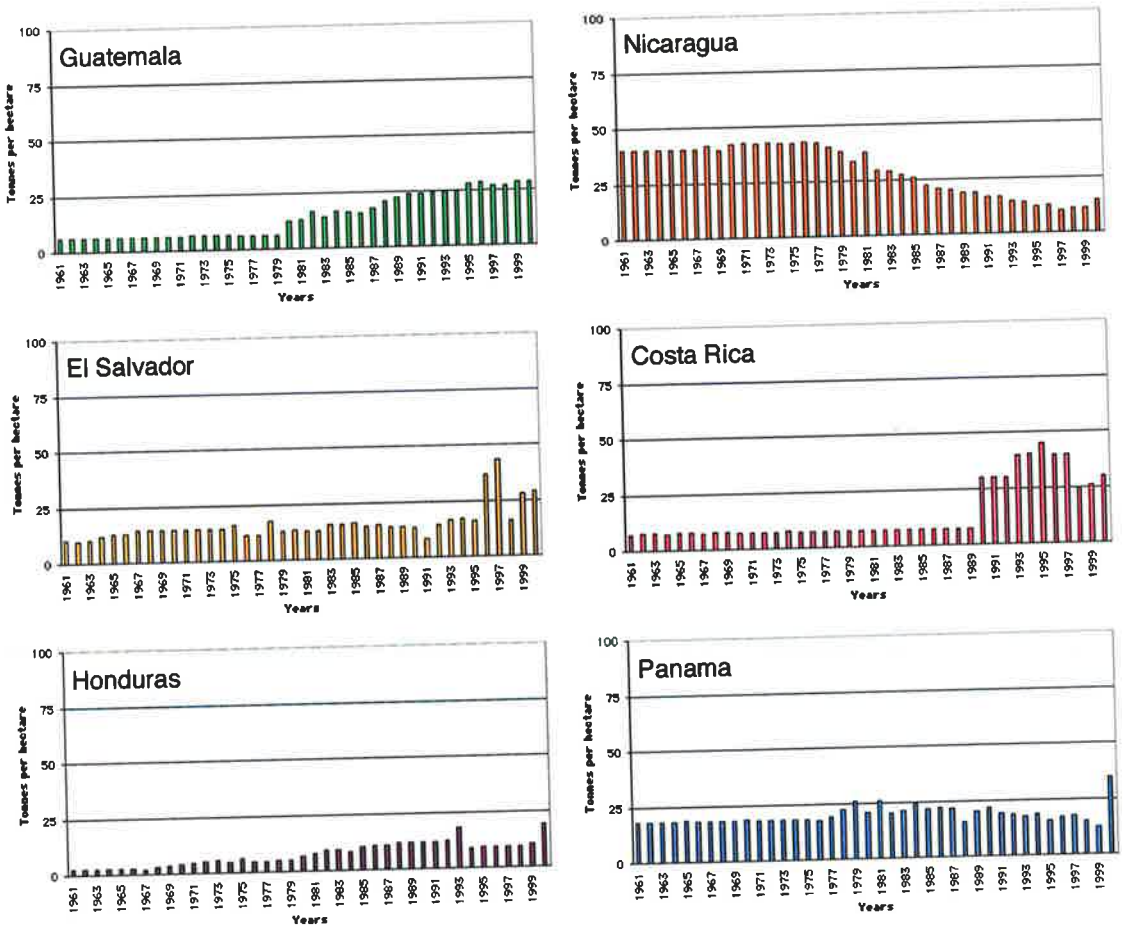


Figure 13. Average yearly tomato production (tonnes per hectare) between 1961 and 2000 for tomato producing regions of Central America (Source: FaoStat).

The geminiviruses that infect tomato in Mexico often occur as variable mixtures of viruses in different regions and seasons (Polston and Anderson, 1997). In addition to viruses associated with rizado amarillo and tigre diseases (Polston and Anderson, 1997), the following viruses endemic to Mexico include:

- *Chino del tomate virus* (Brown and Nelson, 1988), renamed ToLCrV (Fauquet, *et al.,* 2000),
- *Sinaloa tomato leaf curl virus* (Brown *et al.*, 1993; Idris and Brown, 1998), ToLCSinV (Fauquet, *et al.,* 2000),
- *Tomato leaf crumple virus* (Paplomatas *et al.*, 1994), ToLCrV (Fauquet, *et al.,* 2000),
- *Pepper huasteco virus* (Torres-Pacheco *et al.*, 1993; Torres-Pacheco *et al.*, 1996), PHYVV (Fauquet, *et al.,* 2000),
- *Serrano golden mosaic virus* (Brown and Poulos, 1990), now PepGMV (Fauquet, *et al.,* 2000),
- *Texas pepper virus* (Brown and Poulos, 1990), now PepGMV (Fauquet, *et al.,* 2000).
- *Tomato mottle virus* (ToMoV) (Garrido-Ramirez and Gilbertson, 1998).

During the 1970-1971 season, the *Chino del tomate* virus was reported to infect tomatoes along the west coast of the State of Sinaloa in Mexico (Brown and Nelson, 1988). This virus also caused local disease epidemics during the period of 1976 to 1983 and in some years after 1988. Since 1990, these viral epidemics coincide with high populations and activity of *B. tabaci* B-biotype whiteflies (Torres-Pacheco *et al.*, 1996). Until recently, *Chino del tomate virus* was commonly found in both pepper and tomato fields in Sinoloa, but now it appears to be largely displaced by other

geminiviruses including *Pepper huasteco virus*, *Serrano golden mosaic virus*, *Sinaloa tomato leaf curl virus* and *Texas pepper virus* (Polston and Anderson, 1997). *Chino del tomate virus* has also been detected in tomato in the Mexican states of Chiapas, Morelos and Tamaulipas with *Pepper huasteco virus* and *Texas pepper virus* (Torres-Pacheco *et al.*, 1996). *Pepper huasteco virus* has been found widely distributed in pepper and tomato crops throughout Mexico and *Texas pepper virus* is known to have caused tomato disease epidemics in Tamaulipas (Torres-Pacheco *et al.*, 1996). The variation in yearly average tomato production in Mexico, USA and Canada is shown in Figure 14.

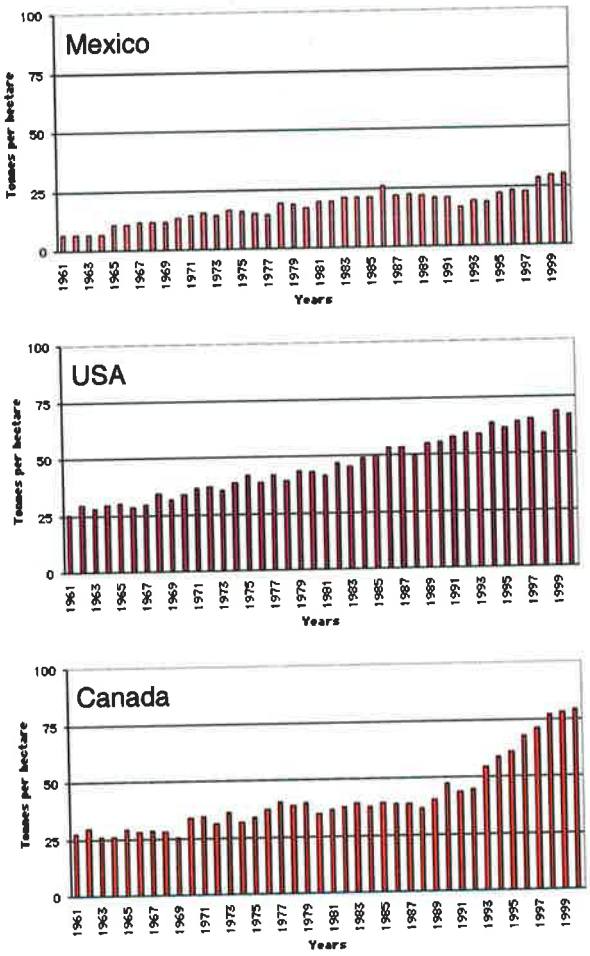


Figure 14. Average yearly tomato production (tonnes per hectare) in Mexico, USA and Canada during the period from 1961 and 2000 (Source: FaoStat).

Geminiviruses have not been reported to cause problems in Canada. In the USA, begomoviruses first caused problems for tomato production in Texas when high populations of *Bemisia tabaci* biotype B were associated with viral epidemics caused by *Texas pepper virus* in tomato and pepper during 1987 (Stenger *et al.*, 1990). These viral epidemics in the Texas lasted for a few consecutive years and have not been recorded since (Polston and Anderson, 1997). Sporadic occurrences of *Bemisia tabaci* in Florida were first recorded there in the late 1800's but for many years they were not regarded as important pests of the tomato crop (Polston and Anderson, 1997). By 1987, high populations of *Bemisia tabaci* whiteflies were observed to infest tomato plants causing some concern (Schuster *et al.*, 1990). During 1989, the first incidences of virus-like symptoms in tomato were observed (Simone *et al.*, 1990; Kring *et al.*, 1991). Subsequently, the cause of this disease was identified as ToMoV (Abouzid *et al.*, 1992; Polston *et al.*, 1993; McGovern *et al.*, 1994). By the end of the growing season in 1992-93, the infection rates of ToMoV-infected tomato plants in some fields were recorded as high as 100% (Polston *et al.*, 1996). ToMoV is believed to have originated within Florida and occurrences outside this state probably resulted from movement of ToMoV-infected plants from Florida (Polston and Anderson, 1997). Epidemics of ToMoV in tomato have occurred in the States of South Carolina and Tennessee and a few infected plants have been found in the state of Virginia (Polston *et al.*, 1995).

Recently, TYLCV has been detected in tomato fields in Florida and southern Georgia (Momol *et al.*, 1999; Polston *et al.*, 1999). The disease symptoms caused by the virus were first observed in a few counties of Florida during 1997. The virus was detected in tomato plants collected from retail outlets and from home gardens. The virus-infected plants were traced back to production facilities in Dade County (Polston *et al.*, 1999).

The establishment of TYLCV in Dade County is now a concern for both tomato producers and retail plant producers. The eradication of this virus from Florida is unlikely. Recurrences of TYLCV infection can be expected at least in Dade County and reintroduction to other areas of Florida remain a risk (Polston *et al.*, 1999). It seems likely that this virus will cause significant loss of tomato crop in Florida as it becomes more widespread.

1.8.2.4 Australia

The average yearly tomato production per hectare in Australia has more than doubled during the period from 1961 to 2000 (Figure 15). In 1998, total production reached 393,100 tonnes with a gross value estimated to be worth $A177 million (Australian Bureau of Statistics).

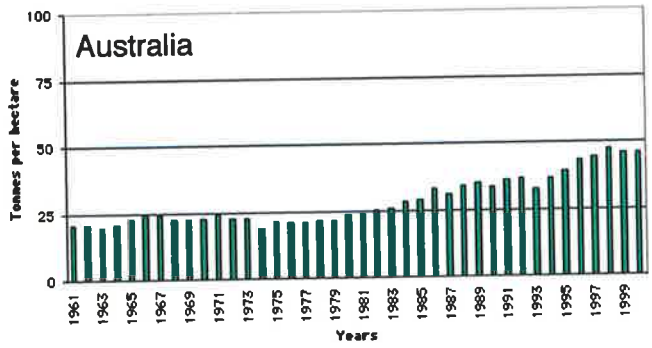


Figure 15. Average yearly tomato production per hectare during the period from 1964 to 1997 (FaoStat).

In Australia, the occurrence of begomovirus-induced tomato disease has so far been limited to isolated occurrences in northern Australia. The presence of tomato leaf curl disease has been observed each year near Darwin in the Northern Territory since 1970 (Condé and Connelly, 1994). This whitefly-transmitted disease has sometimes caused severe damage to early and mid-season tomato crops in coastal areas (Figure 16). Other isolated occurrences of the disease have been recorded at Kowanyama, Queensland (Thomas *et al.*, 1986), Karumba, Queensland during 1998 (Figure 17A, B), and some areas of north-east Queensland (M. A. Rezaian, personal communication). The recent arrival of the silverleaf whitefly in Australia (De Barro, 1995, Gunning *et al.*, 1997) may within a few years influence the distribution pattern of ToLCV, and in time these insects could spread the virus into the commercial tomato growing areas of Queensland and New South Wales.

Figure 16. Occurrence of tomato leaf curl virus disease in early and mid-season tomato plantings near Darwin in the Northern Territory (Photos provided by M. A. Rezaian).

A virus isolated from plants with tomato leaf curl disease from Darwin has been shown to be a begomovirus (ToLCV) with a monopartite single-stranded DNA genome of 2766 nucleotides (Dry *et al.*, 1993). Identical disease symptoms were reproduced in plants inoculated with a dimeric clone of ToLCV-DNA (Figure 17C). Subsequently, two further related isolates of ToLCV have been also sequenced from infected tomato and shown to have 97 and 98% sequence similarity to the original isolate (Behjatnia *et al.*, 1996).

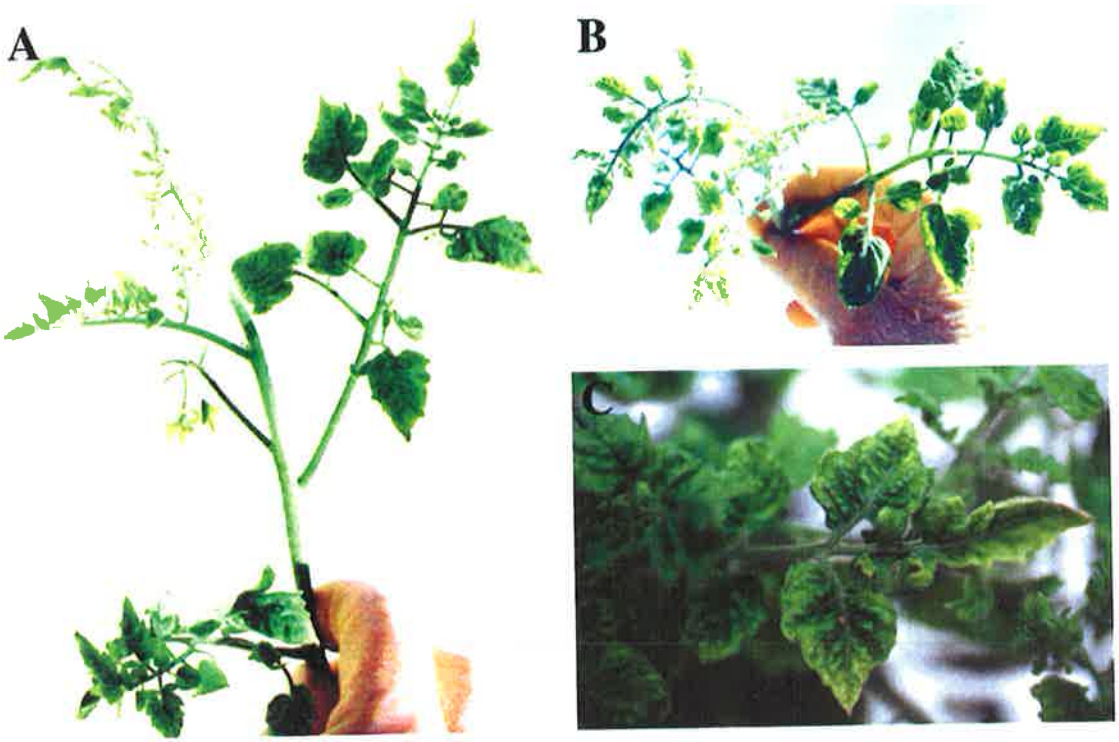


Figure 17. Examples of tomato leaf curl disease from Karumba, northern Queensland (A, B) and symptoms induced in tomato (variety Gross Lisse) by ToLCV-DNA (C).

Indigenous *Bemisia tabaci* whiteflies are suspected to transmit ToLCV from infected native *Solanum* species to tomato plants. Plants such as *S. melongena, Datura stramonium, Nicotiana tabacum* and *Zinnia elegans* were shown to be alternative hosts of ToLCV (Condé and Connelly, 1994). Native *S. pugiunculiferum* growing near Karumba, northern Queensland (Figure 18) was also found to be naturally infected with ToLCV (L. Krake, M. Gunther and M. A. Rezaian, unpublished data).

Figure 18. Native *Solanum pugiunculiferum* from Karumba, northern Queensland naturally infected with tomato leaf curl virus (ToLCV).

1.9 Molecular strategies used for viral-induced resistance

1.9.1 Genetic transformation of plants

The ability of *Agrobacterium* species to transfer and integrate T-DNA into the plant cell has been modified and adapted for the introduction of foreign DNA into plants (Fraley *et al.,* 1983; DeBlock *et al.,* 1984; Horsch *et al.,* 1985). The removal of the *onc* genes from the T-DNA of the Ti-plasmid has permitted its use for genetic transformation. Binary vectors are a further development of T-DNA based vectors and variations of these have been used extensively. These modified vectors are based on the observation that the 25-bp borders of the T-DNA are all that are required to transfer the T-DNA segment into the plant genome, provided that the transfer capability from the *vir* region is supplied in *trans* (Hoekema *et al.,* 1983). A wide variety of binary vectors are now available (Bevan, 1984; Deblaere *et al.,* 1985; Klee *et al.,* 1985; An *et al.,* 1986; Becker *et al.,* 1992). Many of these vectors contain polylinkers to give flexibility and ease in cloning, additional selectable markers or reporter genes to screen transformants, and expression cassettes to measure differing levels of gene expression.

Although direct gene transfer methods are also used, the precision with which the *Agrobacterium* transfers its DNA into the host plant genome makes it ideal for general use in genetic transformation. Successful genetic transformation of plants depends on the stable integration of the introduced DNA into the genome of a cell and the ability to regenerate whole plants from a selected transformed cell line. The integrated DNA is normally randomly inserted into the nucleus, although transformation of the chloroplast is also possible (De Block *et al.,* 1985). Unique transformation events produce the individual transgenic plant lines, some of which may have multiple inserts at different loci within the genome (Zambryski *et al.,* 1989). Analysis of plant progeny regenerated

from *Agrobacterium*-mediated transformation has shown that the inserted genes can be inherited in the subsequent plant generations, although in some situations genetic instability has been observed (Gheysen *et al.,* 1990). The ideal transformation system is one where all plants regenerated from non-transformed cells are identical to parental lines and free from genetic instability resulting from somaclonal variation (Scowcroft and Larkin, 1982). The ultimate aim is to regenerate plants identical to the parental source except for the effect of the newly inserted gene(s).

The first application of genetic transformation to the study of geminiviruses was the introduction of single and tandem direct-repeat copies of TGMV DNA-A and DNA-B molecules into the chromosomes of petunia plants (Rogers *et al.,* 1986). Although the resulting transgenic plants appeared normal, the analysis of DNA extracted from trangenic plants showed free circular single and double-stranded viral DNA's in the plants with a dimeric insert of the DNA-A component. None of the plants transformed with a monomeric insert of DNA-A or monomeric or multimeric inserts of DNA-B produced replicating viral DNA. Since, the transformation method has been used widely to transfer genes into cells and methods are now available for the regeneration of plants from tissues of plant species including important agricultural crops (Lal and Lal, 1990).

1.9.2 Pathogen-derived resistance

The concept of pathogen-derived resistance (Sanford and Johnston, 1985) was first demonstrated for viruses in transgenic plants expressing the coat protein gene of tobacco mosaic virus (Powell-Abel *et al.*, 1986). The mechanism responsible for delaying the onset of virus-induced disease in transgenic plants following challenge-

inoculation was termed coat protein-mediated resistance. Since this demonstration, the coat protein of other viruses has been expressed as transgenes in a variety of model test plants and crops. A range of resistance has been demonstrated in transgenic plants ranging from immunity to susceptibility to homologous and closely related viruses. Within the virus-inoculated population of transgenic plants, there were also some plants that tolerated lower levels of viral replication resulting in the disease symptoms of these plants being delayed by days or weeks when compared with plants infected with wild-type viruses. The general findings of the research indicate that resistance obtained with coat protein gene constructions cannot be explained on the basis of one mechanism operating alone (Kaniewski and Lawson, 1998). In addition to coat protein genes, other genes from plant viruses have been expressed in transgenic plants either as full-length products or as truncated products and shown to interfere with virus replication and the infection cycle. Genes that have an essential replication function or serve as essential movement proteins have been used with some success (Carr and Zaitlin, 1993; Donson *et al.*, 1993; Cooper *et al.*, 1995).

Expression of antisense RNA in plants has been associated with successful suppression of endogenous genes (Sheehy *et al.*, 1988; Smith *et al.*, 1988; van der Krol *et al.*, 1988; Rothstein and Lagrimini, 1989; Schuch *et al.*, 1989; Mol *et al.*, 1990; Gray *et al.*, 1992). In view of the efficient down-regulation of gene expression by mRNA-directed antisense RNAs, it was widely predicted that replication of plant viruses may also be suppressed by antisense RNAs directed against the pathogenic viral RNA. In practice, the low efficiency of antisense RNA as an antiviral agent contrasts with the effectiveness of antisense RNA in gene suppression (Tabler *et al.*, 1998). It was suggested that multicopy transgenes are required with an inverted repeat arrangement

so that transcriptional read-through will produce mRNA that could hybridise with the sense transgene mRNA. The expression of excess untranslatable or truncated sense RNA in the cell appears to stimulate a cellular process for sequence-specific removal of RNAs. This mechanism has been shown to give resistance against some plant viruses (Lindbo and Dougherty, 1992; Lindbo *et al.*, 1993; Dougherty *et al.*, 1994; Smith *et al.*, 1994; Swaney *et al.*, 1995; Goodwin *et al.*, 1996). More recently, it has been proposed and demonstrated that simultaneous expression of sense and antisense RNA in transgenic plants forming a double-stranded region can induce post-transcriptional gene silencing (Waterhouse *et al.*, 1998). Other proposed strategies include various mechanisms based on ribozyme technology. These too have also been used against plant viral RNAs, both *in vitro* and *in vivo* and continue to undergo further development (Lamb and Hay, 1990; De Feyter *et al.*, 1996).

1.9.3 Application as a strategy to combat geminiviruses

The expression of antisense RNA targeted against the *ac1* gene of TGMV efficiently inhibited viral DNA replication in tobacco leaf discs derived from transgenic plants expressing the antisense RNA to the TGMV ORF AC1 (Day *et al.*, 1991). These transgenic tobacco plants also showed less severe disease symptoms following agro-inoculation of the plants with wild-type TGMV compared to the inoculated control plants. The observed resistance in the inoculated plants correlated with higher antisense RNA levels in transgenic plants (Day *et al.*, 1991). An extension of this study showed a four-fold reduction in the replication of BCTV viral DNA in transgenic tissues expressing TGMV antisense *ac1* RNA, but the same transgenic tissues did not influence the level of ACMV DNA replication (Bejarano and Lichtenstein, 1994). While the equivalent *ac1* regions of BCTV and ACMV showed similar overall

homology to that of TGMV (63% and 64%, respectively), within this region, BCTV has a 280 nucleotide stretch of 82% homology to TGMV. In contrast, the homology of this stretch in ACMV is more dispersed. It was proposed that a critical region of complementarity is needed to allow duplex formation to block expression of the target mRNA (Bejarano and Lichtenstein, 1994). The application of the introduction of antisense RNA of the *rep* gene into transgenic plants to interfere with the replication of geminiviruses has been extended to TYLCV (Bendahmane and Gronenborn, 1997). In this study, the inoculated transgenic *N. benthamiana* expressing antisense *c1* RNA displayed a spectrum of symptoms ranging from severely diseased through to the virtual absence of visual symptoms. The observed resistance of two lines was shown to be effective through at least two generations.

The expression of the *rep* gene from ACMV under the control of an enhanced cauliflower mosaic virus 35S promoter in transgenic *N. benthamiana* plants was shown to confer virus-specific resistance against ACMV infection (Hong and Stanley, 1996). The majority of the mechanically inoculated plants remained asymptomatic or produced delayed symptoms and accumulated reduced levels of viral DNA in comparison to the infected control plants. The *rep* gene contains several conserved domains including the nucleoside triphosphate (NTP)-binding and DNA-nicking domains. Gene constructions with function-abolishing mutations in the NTP-binding or DNA-nicking domains were shown to induce *trans*-dominant inhibition of BGMV-DNA replication (Hanson and Maxwell, 1999).

Defective subgenomic DNA species have been reported to occur naturally in plants infected by geminiviruses (Stanley and Townsend, 1985; MacDowell *et al.*, 1986;

Coutts and Buck, 1987; MacDonald *et al.*, 1988; Roberts *et al.*, 1988; Czosnek *et al.*, 1989; Stanley *et al.*, 1990; Frischmuth and Stanley, 1991b; Frischmuth and Stanley, 1992; Stenger *et al.*, 1992; Frischmuth *et al.*, 1997; Sharma *et al.*, 1997; Stanley *et al.*, 1997). When cloned and reintroduced into *N. benthamiana* by co-inoculation with both genomic components of ACMV, the subgenomic DNA interfered with virus multiplication causing reduction in the number of infected plants and attenuation of symptoms, raising the possibility that subgenomic molecules may behave as defective interfering DNA (Stanley and Townsend, 1985). Transgenic *N. benthamiana* plants transformed with subgenomic DNA-B of ACMV produced plants that displayed ameliorated virus symptoms following challenge inoculation with virus or cloned DNA (Stanley *et al.*, 1990). The episomally replicated subgenomic DNA was amplified 3-fold over the level of the full-length DNA-B. Serial infection of transformants resulted in a further decrease in symptom severity and viral DNA levels. However, these transgenic plants did not resist infection when challenged with BCTV or TGMV, thus showing the specific nature of the interfering mechanism (Stanley *et al.*, 1990; Frischmuth and Stanley, 1991b). An additional study with transgenic *N. benthamiana* plants transformed with a partial-repeat of a cloned defective subgenomic circular DNA species of BCTV also displayed ameliorated disease symptoms following agro-inoculation with BCTV (Frischmuth and Stanley, 1994). Analysis of viral DNA from the plants challenged with the wild-type virus showed only 10-30% of the level of viral DNA detected in the infected nontransformed control plants showing normal infection. Likewise, transgenic *N. benthamiana* plants with integrated tandem repeat copies of BCTV DI-DNA derived from the Logan strain of BCTV were shown to display attenuated symptoms following challenge-inoculation with the homologous virus (Stenger, 1994). In contrast, these transgenic plants showed no resistance when

challenged with the CFH and Worland strains of BCTV and no mobilisation or amplification of the integrated Logan DI-DNA occurred, showing the existence of specificity among strains of BCTV. This interfering mechanism may have an application against other geminviruses where less variation exists between strains.

The coat protein mediated mechanism was reported to give resistance to the whitefly-transmitted TYLCV in transgenic tomatoes (Kunik *et al.*, 1994). These transgenic F1 tomato plants expressing the viral coat protein were inoculated with TYLCV using whitefly-mediated transmission. In some cases, the inoculated transgenic plants expressed delayed disease development and recovery from disease following repeated inoculation. Similarly, tobacco plants (*N. tabacum* var. Xanthi) were transformed with a binary vector containing a modified *cp* gene of ToMoV with a deletion of 30 nucleotides at the 5'-end (Sinisterra *et al.*, 1999). The response of plants to high inoculum levels of viruliferous whiteflies varied and ranged from susceptible to immune. Although the transgene RNA was detected in the immune plants, no translation product could be detected, indicating the viral resistance of the plants may be mediated by the transgene transcript.

Ribosome-inactivating proteins that occur naturally in a variety of plant species have also been tested for antiviral activity. The pokeweed antiviral protein and dianthin from *Dianthus caryophyllus* have been shown to exhibit antiviral activity against a broad range of plant viruses when applied exogenously to inoculated leaves or when expressed constitutively in transgenic plants (Chen *et al.*, 1991; Lodge *et al.*, 1993; Taylor *et al.*, 1994). Dianthin has been expressed in transgenic *N. benthamiana* from the ACMV virion-sense promoter that is *trans*-activated by the product of the viral *ac2*

gene (Hong *et al.*, 1996; Hong *et al.*, 1997). This system has been developed to produce phenotypically normal plants and to ensure that transgene expression is localised to virus-infected cells. When *N. benthamiana* transgenic plants were challenged with ACMV, the inoculated leaves produced atypical necrotic lesions indicative of dianthin expression (Hong *et al.*, 1996). The ACMV inoculated plants accumulated less viral DNA and displayed attenuated systemic symptoms with recovery but these transgenic plants failed to show resistance against four other geminiviruses.

Wild-type and mutant versions of the *bc1* gene of ToMoV (Duan *et al.*, 1997a: Duan *et al.*, 1997b) have also displayed delays in the development of disease symptoms following challenge-inoculation with the homologous virus. Likewise, the same effects were achieved when transgenic tomato plants expressing *bc1* from BDMV were challenge-inoculated with ToMoV (Hou *et al.*, 2000).

1.10 The aim of the following study

The work described in this thesis has been part of a larger project with the aim to understand the basic biology of tomato leaf curl virus with the ultimate goal to develop molecular strategies for induced resistance in tomato against infection with geminiviruses. The research presented here had three specific objectives:

1. To produce transgenic tobacco and tomato plants expressing the *c4* gene from ToLCV under the control of a 35*S* promoter/terminator from cauliflower mosaic virus and to evaluate the phenotypic responses.

2. Prepare a frame-shift modified version of the 35*S*-*c4* gene construction and produce transgenic tobacco and tomato plants expressing the modified gene for direct comparison with plants expressing the ectopic *c4* gene.

3. Assess the transformed plants for resistance against challenge-inoculation with infectious ToLCV-DNA.

Chapter 2

Materials and Methods

2.1 Reagents

The chemicals used in all experiments were analytical grade or molecular biology quality. These chemicals were obtained from the following companies: Ajax Chemicals (Auburn, N.S.W.), BDH Chemicals, Australia (Killsyth, Victoria), May & Baker Ltd. (Dagenham, England), FMC Bio Products (Rockland, USA), Pharmacia (Uppsala, Sweden) and Sigma Chemical Co (St. Louis, USA). The enzymes used were obtained from Boehringer Mannheim Australia (Castle Hill, N.S.W.) and Promega Corporation (Annandale, N.S.W.). Specific molecular biology kits were obtained from GeneWorks (Adelaide), Bio 101 (La Jolla, California, USA), Promega, and Qiagen (Hilden, Germany). All antibiotics and the ingredients of culture media were supplied from Sigma Chemical Co and Difco Laboratories (Detroit, USA).

2.2 Source of ToLCV-DNA

The original source of tomato leaf curl disease was obtained from a field planting of tomato (*Lycopersicon esculentum* L.) near Darwin, Northern Territory, and supplied by Mr B. Conde (Department of Primary Industry and Fisheries, Berrimah, Northern Territory, Australia). Diseased plants were maintained by grafting 2-node shoot sections of the infected tomato onto the tomato cultivar Grosse Lisse. Cloned full-length DNA from *Tomato leaf curl virus* (Dry *et al.*, 1993) was used as the base material for gene constructions.

2.3 Cultivation of plants

All virus-infected plants were confined to a glasshouse compartment modified to conform to regulations specified by the Australian Quarantine Inspection Service (AQIS). Genetically-modified plants were contained in a separate glasshouse compartment under conditions specified by the Genetic Manipulation Advisory Committee (GMAC, Canberra, ACT).

All plants were grown in a U.C.-type potting mix (Matkin and Chandler, 1957), comprising 2 parts of coarse river sand and 1 part peat moss, amended with 0.37% (w/v) dolomite, 0.1% (w/v) gypsum, 0.1% (w/v) lime, to give a soil pH of 5.8. Plants were supplemented with Osmocote slow-release fertilizer (Scotts Aust. Pty. Ltd.), and with weekly applications of liquid Aquasol (Hortico Aust. Pty. Ltd.).

2.4 *In vitro* tissue culture

The tissue culture medium was autoclaved at 121°C for 20 minutes or filter-sterilized through a 200 µm filter (Sartorius, Gottingen, Germany). All manipulations were done under aseptic conditions in a laminar-flow cabinet (Gelman Sciences Australia). Media were prepared from 0.43% Murashige and Skoog salts (Sigma Chemical Co) supplemented with 3% sucrose, 0.1% Gamborg's vitamin mixture (Sigma Chemical Co) and adjusted to pH 5.7 with 0.1N KOH. When a solid medium was required, either 0.8% (w/v) Phytagel (Sigma Chemical Co) or 0.7% (w/v) Bacto-agar (Difco Laboratories) was added prior to autoclaving. Additional components (Sigma Chemical Co) shown in Table 11 were added when required after the autoclaving process.

Table 11. Additives used to modify the composition of the culture media

Additives	Final concentration in media
BA plus NAA	1 µg ml^{-1} of BA and 0.1 µg ml^{-1} of NAA
Kanamycin	50, 100 and 250 µg ml^{-1}
Cefotaxime	500 µg ml^{-1}
IAA aspartic acid	0.872 µg ml^{-1}
Zeatin riboside	1.748 µg ml^{-1}
Carbenicillin	200-500 µg ml^{-1}

Seeds of tobacco (*Nicotiana tabacum* var. Samsun) or tomato (*Lycopericon esculentum* var. Ailsa Craig) were sterilised in 4% sodium hypochlorite for 30 minutes. The sterilised seeds were rinsed six times with sterile water before transfer onto 90 x 20mm deep petri-dishes or 153 x 70mm diameter vials containing sterilised 0.43% Murashige

and Skoog medium, 3% sucrose, 0.1% Gamborg's vitamins, pH 5.7 and solidified with 0.7% Bacto-agar.

2.5 Molecular cloning techniques

Stock solutions (Table 12) were prepared with water purified with the NANOpure system (Barnstead) at greater than 11.5 megohm-cm. These solutions were autoclaved at 121°C for 20 minutes, or prepared using a flamed spatula, sterile glasswareware or plasticware and sterile NANOpure water. Instructions and protocols used were essentially as described by (Sambrook *et al.*, 1989). All antibiotics were stored at -20°C.

Table 12. Stock solutions

Solutions	Composition
Ampicillin	Sterile-filtered 2% (w/v) ampicillin,
Gel-loading buffer	78% glycerol, 1.2 mg ml^{-1} ribonuclease A, 0.25% (w/v) bromophenol blue, 0.25% xylene cyanol, 1 mM EDTA
IPTG	Sterile-filtered 20% (w/v) IPTC
Kanamycin	Sterile-filtered 5% (w/v) kanamycin
Luria-Bertani medium plus glucose	1% (w/v) Bacto-tryptone, 1% (w/v) NaCl, 0.5% (w/v) yeast extract, 0.1% (w/v) glucose
Rifampicin	Sterile-filtered 2.5% (w/v) rifampicin in DMSO
Solid Luria-Bertani medium	Autoclaved Luria B medium plus 1.2% (w/v) Bacto-agar
TE buffer, pH 8.0	10 mM Tris-HCl pH 8.0, 0.1 mM EDTA pH 8.0
X-gal	2% (w/v) X-gal in dimethylformamide
1 X TBE buffer	89 mM Tris-Borate pH 8.3, 2 mM EDTA
1 x Agar–gel solutions	0.8 or 1 or 1.2% Type 1-A agarose in 1 x TBE buffer
20 x SSC, pH 7.6	3 M NaCl, 0.3 M sodium citrate

2.5.1 Bacterial cultures and plasmid vectors

The bacterium *Escherichia coli* strain DH5α (Stratagene, La Jolla, California, USA) was used in all sub-cloning steps to replicate each of the specified vectors. These vectors included pBluescript SK^+ (pJIT 163, Guerineau *et al.*, 1992) and the binary vector pBin19 (Bevan, 1984; Frisch *et al.*, 1995). The pBluescript SK^+ (Stratagene) was selected on the basis of its multiple cloning sites, ampicillin resistance gene, T3, T7 promoters and the *lacZ* gene to provide α-complementation for blue/white selection of bacterial colonies. The vector pJIT163 containing a tandem 35S promoter from cauliflower mosaic virus (CaMV), multiple cloning sites, and CaMV 35S terminator was used to produce p35*S-c4*. In a later sub-cloning step, pBin 19 with kanamycin resistance was used as the binary vector. *Agrobacterium tumefaciens* strains C58 and LBA4404 were also used to propagate the binary vector.

Dr Ian Dry generously supplied the initial frozen stocks of bacterial cells in 20% (v/v) glycerol. Bacterial cultures were grown in Luria-Bertani (LB) medium (Sambrook *et al.*, 1989) supplemented with 0.1% (w/v) glucose and an antibiotic appropriate for the bacterial vector used. Stock antibiotic solutions and LB medium were prepared as indicated in Table 2. All operations with bacteria were performed under aseptic conditions.

2.5.1.1 Culture of *Escherichia coli*

Recombinant *E. coli* strain DH5α cells were cultured by inoculating a single bacterial colony into a tube containing 2ml of liquid LB medium with glucose and an appropriate antibiotic. The antibiotics used included ampicillin at a final concentration of 100 $\mu g\ ml^{-1}$ and kanamycin at 50 $\mu g\ ml^{-1}$. Each culture was grown overnight at 37°C with constant agitation at 300 rpm.

Culture plates used to grow bacterial colonies with ampicillin or kanamycin resistance were prepared from liquid LB plus glucose solution containing 1.2% (w/v) Bacto-agar. This medium was autoclaved and cooled to 55°C before the addition of 100 μg ml^{-1} ampicillin or 50 μg ml^{-1} of kanamycin. The medium was poured to cover the bottom-surface of 14 mm diameter Petri dishes. The culture plates used for blue/white colony selection were prepared as described above, but additionally contained 25 μg ml^{-1} of IPTG and 40 μg ml^{-1} of X-gal. All plates spread with bacterial cells were incubated overnight at 37 °C.

2.5.1.2 Culture of *Agrobacterium tumefaciens*

A 2.5 ml liquid culture of *A. tumefaciens* strain C58 or LBA4404 containing a recombinant pBin19 plasmid was prepared from a single bacterial colony inoculated into LB plus glucose medium supplemented with 50 μg ml^{-1} of kanamycin and 50 μg ml^{-1} of rifampicin. Each overnight culture was incubated at 28°C, with constant shaking at 300 cycles min^{-1}. Culture plates were prepared from LB plus glucose and 1.2% (w/v) Bacto-agar before the addition of 50 μg ml^{-1} of kanamycin and 50 μg ml^{-1} of rifampicin. The inoculated plates were incubated at 28°C for 2 days.

2.5.1.3 Glycerol stocks of bacterial cultures

Long-term storage of bacterial cultures was achieved by mixing 0.9 ml of a bacterial culture in the log-growth-phase with an equal volume of sterile 40% glycerol. This mixture was snap-frozen and stored at -70°C.

2.5.2 Preparation of bacterial plasmids

2.5.2.1 Small-scale plasmid preparation

Plasmids were routinely isolated using the rapid boiling method (Holmes and Quigley, 1981). Overnight cultures of 2-2.5 ml were centrifuged at 5600 *g* in a Micro Centaur (MSE) centrifuge to pellet the bacterial cells. The pellets were drained and resuspended in

0.35 ml of STET medium (8% w/v sucrose, 5% v/v Triton X-100, 50 mM EDTA, 50 mM Tris-HCl pH 8). An aliquot of 12.5 μl of lysozyme solution (2% w/v lysozyme in 10 mM Tris-HCl pH 8) was added before the mixture was placed in a water-bath at 100°C for 1 minute (*E. coli* cells) or 45 seconds (*A. tumefaciens* cells). The lysed cells were centrifuged at 11,300 *g* for 30 minutes. The gelatinous precipitate was removed with the aid of a toothpick and the remaining nucleic acid precipitated by adding 40 μl of 3 M sodium acetate, pH 5.0 and 220 μl of cold isopropanol. Following centrifugation, the nucleic acid pellet was drained, rinsed with cold 80% ethanol and dried *in vacuo*. The pellet from each recombinant *E. coli* culture was resuspended in 30 μl of sterilized water.

The pellet from each *A. tumefaciens* culture was resuspended in 200 μl of TE pH 8, and subjected to further purification including two separate extractions with phenol-chloroform followed by extraction with chloroform before ethanol precipitation. The final pellet was drained, washed with 80% ethanol, dried *in vacuo* and resuspended in 20 μl of water.

2.5.2.2 Larger-scale plasmid preparation

The isolation of plasmid from a 50 ml overnight bacterial culture was achieved using a Plasmid Kit (Qiagen). The bacterial cells were precipitated by centrifugation at 6,000 *g* for 2 minutes and processed according to the manufacturer's instructions.

2.5.3 Restriction enzyme digests of DNA

Plasmid DNA was digested with restriction endonucleases using buffer solutions supplied by the manufacturers. Generally, 1-4 μg of recombinant DNA was digested for 1-2 hours at 37°C with 10-20 units of enzyme in a 20 μl reaction. An appropriate concentration of resuspended Agarose in 1X TBE buffer containing 0.5 μg ml^{-1} ethidium bromide was used to prepare a gel in an Easy-Cast Electrophoresis apparatus, Model

B1A (OWL Scientific Inc.) or a MUPID apparatus (Advance Co. Ltd., Japan). TBE buffer was added to serve as the electrolyte during electrophoresis.

Each aliquot of enzyme reaction to be analysed by electrophoresis was mixed with 0.2% volume of gel loading buffer (Table 12) and loaded in one of the slots formed during the casting of agarose-gels. An appropriate set of DNA markers (Promega) was loaded into a slot adjacent to the samples to be analysed. Electrophoresis was performed under constant current conditions. The separated DNA bands were viewed on an UV transilluminator (NovaLine) and the results were recorded photographically. Recovery of selected DNA bands was achieved by replacing unwanted agarose-gel with freshly-cast 1.5% NuSieve GTG (FMC) gel in TBE buffer and continuing electrophoresis until the selected DNA band migrated into the low-melting-point NuSieve gel. A small strip of this gel containing the selected DNA band was cut from the gel and stored at -20°C.

2.5.4 Dephosphorylation of restricted vector DNA

Calf intestinal alkaline phosphatase (Boehringer Mannhein) was used to remove the 5'-phosphate group from the ends of linearised vector to prevent self-ligation. A 50 or 60 μl reaction volume containing vector DNA, 1x dephosphorylation buffer (Boehringer Mannhein), and 1 unit of calf intestinal alkaline phosphatase (CIP) were incubated at 37°C for 30 minutes before incubation with 1 mg ml^{-1} ribonuclease A at 37°C for a further 30 minutes. Each reaction was purified using phenol-chloroform extraction followed by chloroform extraction and ethanol precipation, or by the use of a QIAquick Nucleotide Removal Kit (Qiagen) according to the manufacturers instructions.

2.5.5 End-filling of DNA fragments

DNA fragments were end-filled to give blunt-ends using T4 DNA polymerase (Boehringer Mannheim) with the incubation buffer supplied by the manufacturer. Each 20 μl reaction additionally contained 250 μM of mixed dNTPs and the purified target

DNA. Each reaction mixture was incubated at 37°C for 30 minutes and the end-filled DNA was gel-purified by electrophoresis through a 1.5% NuSieve GTG agarose (FMC, USA) gel. The band containing the end-filled DNA was cut from the gel and stored at -20°C.

2.5.6 Ligation of DNA

Linearised vector (20-50 ng) was either self-ligated or ligated (1:3 molar ratios) with a purified DNA insert. In either case, a piece of NuSieve gel containing the purified selected band of insert DNA or linearised vector was melted by heating at 67°C for 10 minutes, cooled to 37°C for 5 minutes before mixing with 6.5 μl of sterile water. The volume of this reaction mixture was increased to 15 μl with additions of the vector or DNA insert, 0.1% (v/v) T4 DNA ligase buffer and 1 unit of T4 DNA ligase (Boehringer Mannheim) with 1.3 mM ATP. This mixture was incubated at 37°C for 30 seconds, then placed in an insulated block and allowed to slowly cool to room temperature during a period of 1 hour. The mixture was re-heated at 67°C for 5 minutes and cooled to 37°C before electroporation into bacterial cells.

2.5.7 Purification of DNA

Crude DNA was extracted with an equal volume of phenol-chloroform (1:1) and centrifuged (11,300 *g* for 10 minutes) to separate the aqueous phase. The recovered aqueous phase was re-extracted with an equal volume of chloroform-isoamyl alcohol (24:1). Following re-centrifugation, the DNA in the recovered aqueous phase was precipitated with the addition of 0.1 volume of 3M sodium acetate pH 5.0 and 2 volumes of cold ethanol. The DNA precipitate was pelleted by centrifugation (11,000 *g* for 10 minutes), drained, rinsed with 80% cold ethanol, dried *in vacuo*, and resuspended in sterile water.

Other methods were used to purify the DNA from contaminating substances. The Gene-clean method (Bio 101) and the Wizard method (Promega) were used to purify DNA

from bands cut from the agarose gels whereas the QIAquick Nucleotide Removal Kit (Qiagen) was used to purify the plasmid DNA from contaminants after phosphatase reactions. These methods were performed according to the instructions supplied by the manufactures of the kits. Sometimes it was necessary to concentrate the DNA eluates by extraction with n-butanol. This protocol involved the addition of 500 µl of n-butanol to the 50 µl eluate. The mixture was vortexed and centrifuged at 11,300 *g* for 10 minutes. The butanol phase was discarded and the pellet drained, dried *in vacuo*, and resuspended in 5 µl of water.

2.5.8 Electroporation of recombinant plasmids into bacterial cells

2.5.8.1 Preparation of electro-competent cells

The method used to prepare electro-competent cells of *E. coli* was derived from the manual supplied with the Gene Pulser Apparatus (Bio-Rad, Richmond, California, USA) and the method for preparing *A. tumefaciens* cells for electroporation was reported by (Mattanovich *et al.*, 1989). The common steps of both methods are described below together with the variations for the different cell-types.

A 5 ml liquid culture of *E. coli* or *A. tumefaciens* cells incubated overnight was used to inoculate 500 mls of LB plus glucose broth under aseptic conditions. The *E. coli* cells were grown at 37°C, whereas the *A. tumefaciens* cells were incubated at 28°C. These cultures were grown under vigorous constant shaking until an optical density A^{10mm} at 600 nm reached 0.5 units. The cell suspensions were chilled on ice for 10 minutes before centrifugation at 4000 *g* for 15 minutes at 4°C. The supernatant was discarded and the pellets drained. The pelleted cells were gradually resuspended in 500 ml of cold sterile water and re-centrifuged as above. The washed *E. coli* cells were resuspended in 250 ml of cold sterile water while the *A. tumefaciens* cells were washed three times in sterile 1 mM HEPES buffer pH 7.0. Following centrifugation, the pellets were drained and resuspended in 10 ml of cold sterile 10% (v/v) glycerol. The resuspended cells were re-

centrifuged, and the supernatant was replaced with fresh 10% glycerol so that the final volume did not exceed 2 ml. Aliquots of 45 μl of resuspended cells were transferred to 1.5 ml tubes and snap-frozen in liquid nitrogen and stored at -70°C.

2.5.8.2 Electroporation

Electroporation of *E. coli* DH5α and *A. tumefaciens* cells was carried out using a Bio-Rad Gene Pulser apparatus. This machine was set at 25 μf capacitance, 200 ohm resistance, and 1.8 kvolts for *E. coli* cells or 2.5 kvolts for the *A. tumefaciens* cells. An aliquot of 1 μl of ligation mixture was mixed with 40 μl of thawed bacterial cells. The mixture was transferred to a sterile Gene Pulser cuvette with 0.1 mm path length. This cuvette was pulsed according to the manufacturers specification (time constant 3.9-4.2), and 0.6-0.9 ml of liquid LB plus glucose medium mixed with the contents of the cuvette. The liquid mixture was incubated at 37°C for 1 hour (*E. coli*) or at 28°C for 2 hours (*A. tumefaciens*). Aliquots of 5 μl and 200 μl were each spread on to agar-plates containing LB medium, ampicillin or kanamycin, IPTG, and X-gal for selection of recombinant *E. coli* cells (**2.5.1.1**) or onto plates containing LB plus glucose, kanamycin and rifampicin for the selection of recombinant *A. tumefaciens* cells (**2.51.2**). The *E. coli* plates were incubated at 37°C for 16 hours and the *A. tumefaciens* plates incubated at 28°C for 2 days.

2.5.9 Amplification of DNA by polymerase chain reaction (PCR)

2.5.9.1 Extraction of DNA for PCR

Sterile 1.5 ml centrifuge tubes were used as containers for samples. Sterile pestles were prepared by heat-sealing the pointed-end of disposable moulded blue-tips made for the P1000 Gilson Pipeteman. These tips were attached to a Skil Twist (SKIL) cordless screwdriver. Each 25 mg sample of young leaf tissues was tranferred to a 1.5 ml centrifuge tube, taking care to prevent cross-contamination between samples. These samples were snap-frozen in liquid nitrogen. Each sample of frozen tissue was ground to a powder with the assistance of a small amount of sterile acid-washed sand. Ten volumes

of extraction buffer (100 mM Tris pH 8, 25 mM EDTA, 250 mM NaCl, 0.5% SDS, 1% (v/v) ß-mercaptoethanol) was added and mixed. Ten volumes of phenol-chloroform was added and the mixture vortexed. The homogenates were stored on ice until all samples were processed. Each sample was centrifuged and its aqueous phase re-extracted with an equal volume of chloroform-isoamyl alcohol. Following centrifugation, the aqueous phase was transferred to a new tube containing 0.1 volume of 3M sodium acetate pH 5.0 and 1.5 volumes of cold isopropanol. This mixture was stored on ice for 5 minutes before centrifugation to pellet the nucleic acid. Each pellet was drained, rinsed twice with 75% cold ethanol and dried *in vacuo*. Each dried pellet was resuspended in 20 µl of sterile water.

2.5.9.2 Synthetic oligodeoxyribonucleotides

The synthetic oligodeoxyribonucleotides used in this study (Table 13) were derived from sequences of pBin 19 (GenBank accession #U09365) and of ToLCV (GenBank accession #S53251). The C4 ORF primers were generously provided by Dr Ian Dry (CSIRO) and the NPT II and NOS terminator primers were supplied by Elizabeth Lee (CSIRO).

Table 13. Oligonucleotide primers

Identity	Size (nt)	Source	Sequence 5'- 3'
C4 *Hind*III	27	ToLCV, 2466 - 2449	gc gtt cga acc atg aga atg ggg agc c
C4 *Bam*HI	29	ToLCV, 2152 - 2172	gcggatccg ggg cta att ccc taa gga cg
NPT II	21	pBin 19, 8678 - 8657	gaa ggg act ggg tgc tat tgg
NOS	21	pBin 19, 7691 - 7712	atc atc gca aga ccg gca aca

2.5.9.3 PCR conditions

Each PCR reaction (20 μl) contained Promega buffer (50 mM KCl, 10 mM Tris-HCl pH 9.0 at 25°C, 0.1% Triton X-100), 1.5 mM Mg Cl_2, 0.8 μM of each of the two primers, 200 μM of mixed dNTP, 1 μl of prepared DNA template and 0.75 units of *Taq* DNA polymerase (Promega). Each mixture was transferred to an Omn-E thermocycler (Hybaid) with tube control. The first cycle consisted of a denaturation step at 95°C for 3 minutes. The next 30 cycles consisted of a denaturation step at 94°C for 45 (C4) or 55 seconds (NPT II-NOS), followed by annealing at 50°C (C4) or 55°C (NPT II-NOS) for 30 seconds, and extension at 72°C for 2 minutes. The completed 30 cycles was followed by a soak period of 7 minutes at 72°C.

2.5.10 Sequencing of DNA

DNA sequencing was either contracted to services from the Department of Haematology, Flinders Medical Centre, Adelaide, or done manually on site using the dideoxynucleotide chain termination method (Sanger *et al.*, 1977).

The protocols and reagents used for manual sequencing came from the Sequenase version 2.0 DNA Sequencing Kit (United States Biochemical, Cleveland, Ohio, USA). A 20 μl dsDNA strand separation reaction contained 16 μg of template DNA and 2 μl of 2N NaOH. This mixture was applied to a spin-column containing Sepharose G50 (Pharmacia). Following centrifugation, a 6 μl aliquot of the denatured DNA was added to a 10 μl annealing reaction containing 2 μl of Reaction buffer and 2 μl of the M13-20 forward primer. This reaction was heated at 65°C for 2 minutes and slowly cooled to 30°C. The labelling and termination reactions were set-up according to the manufacturers directions using the supplied labelling mix: ddGTP, ddATP, ddTTP, ddCTP, T7 DNA polymerase, enzyme dilution buffer, and α^{35}S dATP. The final reactions were stopped with the addition of the EDTA-xylene cyanol and bromophenol blue dye mix.

A 6% denaturing polyacrylamide gel mixture was prepared with the addition of 7 M urea. Each gel was made using 45 ml of 6% polyacrylamide-7M urea mixture, 45 µl of TEMED and 125 µl of 10% (w/v) ammonium persulphate poured between a pair of taped glass plates separated by 0.2 mm spacers. A sequencing comb (0.2 mm thick) was inserted to form the spaces for loading wells before the gel had polymerized. Following polymerization, the plates containing the acrylamide gel was attached to an electrophoresis apparatus with TBE electrophoresis buffer added and the comb removed. The polyacrylamide gel was preheated by applying 44 mA of current at 1320 volts. The labelling and termination reactions were heated at 80°C for 4 minutes immediately prior to loading each reaction in separate rinsed wells of the preheated gel. The electrophoresis run was continued until the xylene cyanol dye reached the bottom of the gel. The separated plate with gel attached was fixed with a solution containing 5% acetic acid and 15% methanol for 20 minutes. The gel was dried at 70°C, covered with cling-wrap and bands detected by autoradiography.

2.5.11 Extraction and analysis of nucleic acids

2.5.11.1 Extraction of DNA

Samples of 0.1 g or 1 g of young leaf tissue were snap-frozen in liquid nitrogen and stored at -70°C until required for extraction. The composition of the extraction medium was 50 mM Tris pH 8, 100 mM NaCl, 10 mM EDTA, 1% (w/v) SDS, and 1% (v/v) ß-mercaptoethanol. The frozen tissue was pulverized into a powder and mixed with four volumes of extraction medium. Four volumes of buffer-saturated phenol was added and mixed before centrifugation at 11,300 *g* for 10 minutes. The aqueous phase was re-extracted with an equal volume of phenol-chloroform (1:1) until the aqueous phase appeared free of sediment. At this stage, the aqueous phase was re-extracted with chloroform-isoamyl alcohol (24:1). Following centrifugation, the nucleic acids were precipitated in the aqueous phase with 0.1 volume of 3 M sodium acetate pH 5.0 and 1.1

volumes of chilled iso-propanol. Following centrifugation, the pellet was rinsed with 75% cold ethanol, dried *in vacuo,* and resuspended in sterile water.

Each sample of nucleic acid was incubated with 0.25 mg ml^{-1} of ribonuclease A for 30 minutes at 37°C. The digest was extracted with phenol-chloroform (1:1) and the recovered aqueous phase re-extracted with chloroform-isoamyl alcohol (24:1). The DNA was precipitated in the aqueous phase with 0.1 volume of 3M sodium acetate pH 5.0 and 1.5 volumes of chilled isopropanol and pelleted by centrifugation. Each pellet was rinsed with 75% cold ethanol, dried *in vacuo,* and resuspended in sterile water. UV spectrophotometry was used to determine the quality and quantity of extracted DNA.

2.5.11.2 Southern blotting

An aliquot of 20 µg DNA from each sample was adjusted to a 20 µl volume and mixed with 0.2% volume of gel loading buffer. These samples were fractionated through a 1% agarose-TBE gel containing 0.5 µg ml^{-1} of ethidium bromide in a horizontal electrophoresis apparatus (OWL Scientific, Inc., Model B2) at 30 mA of constant current per gel. Gels were photographed on a UV transilluminator (NovaLine), and depurinated in 250 mM HCl for 15 minutes. Each gel was rinsed briefly with water before blotting to a Zeta-probe nylon membrane (Bio-Rad) with 0.4 M NaOH, using the Turboblotter (Schleicher & Scheull) rapid downward transfer system. After blotting, the membrane was washed in 2 x SSC buffer and cross-linked with UV at 120,000 µjoules using a UV Stratalinker 1800 (Stratagene).

2.5.11.3 Alkali dot-blots

Each 50 mg fresh weight sample of young leaf tissue was snap-frozen in liquid nitrogen and homogenized in a 1.5 ml centrifuge tube with 4 volumes of a 1:1 mixture of 1 M NaOH and TE buffer. The homogenate was centrifuged and 5 µl of the supernatant was

spotted onto a piece of Zeta-Probe blotting membrane marked out as a grid. The membrane was washed twice in chloroform, once in 2x SSC and cross-linked with UV.

2.5.11.4 Extraction of RNA

Samples of 100 mg of young leaf tissue were snap-frozen in liquid nitrogen and ground to a powder at 4°C and mixed with 4 volumes of extraction medium (100 mM Tris pH 9, 100 mM NaCl, 10 mM EDTA, 1% SDS, 1% 2-mercaptoethanol). An equal volume of buffer-saturated phenol was added, and the mixture vortexed for 30 seconds. An additional 300 µl of extraction buffer was added to reduce the overall viscosity. The samples were centrifuged at 11,300 *g* for 5 minutes and the supernatant was re-extracted with an equal volume of phenol-chloroform (1:1). Following centrifugation at 11,300 *g* for 10 minutes, the supernatant was re-extracted with an equal volume of chloroform-isoamyl alcohol (24:1). After centrifugation, the supernatant was retained and the nucleic acids precipitated with the addition of 0.1 volume of 3 M sodium acetate pH 5.0 and 1.1 volumes of isopropanol. The nucleic acids were pelleted by centrifugation at 11300 *g* for 10 minutes. The pellets were drained, rinsed with 70% cold ethanol and dried *in vacuo*. Each pellet was resuspended in 30 µl of sterile water. The purity of the nucleic acid was determined by UV spectrophotometry.

2.5.11.5 Northern blotting

A horizontal electrophoresis apparatus (OWL Scientific, model B2) used to fractionate the nucleic acids was pre-rinsed with 200 mM NaOH, 200 mM HCl and sterile water prior to being set-up with a 1.5% agarose-MOPS-formaldehyde gel under denaturing conditions (Sambrook *et al.*, 1989). An aliquot of 20 µg of nucleic acid of each sample was adjusted to a 10 µl volume and mixed with 1.5 volume of a denaturing mix containing deionized formamide: 10x MOPS buffer: formaldehyde (10:2:3), and heated at 55°C for 15 minutes, prior to loading on the gel. A sample of RNA markers (Promega)

mixed with ethidium bromide was treated identically to the samples and loaded in an adjacent well. The gel was run overnight at 20 volts under constant voltage. After electrophoresis, the gel was blotted on to Zeta-probe membrane with 10 x SSC buffer using the Turboblotter rapid downward transfer system. The membranes were washed in 2 x SSC and cross-linked in the UV Stratalinker 1800.

2.5.11.6 Synthesis of ^{32}P-labelled probes

The ^{32}P-labelled probes were synthesized using random decanucleotide primers with a GIGAprime DNA labelling kit (GeneWorks, South Australia). A 24 µl reaction volume contained 25-50 ng of a DNA template which was either a linearised full-length ToLCV-DNA or a 1630 bp fragment of ToLCV-DNA (nt 1075 to 2643) incorporating the complementary-sense genes. The DNA template was heated at 95°C for 5 minutes and immediately cooled on ice. The following ingredients including 6 µl of Nucleotide Buffer Cocktail (160 µM of each of dCTP, dTTP, dGTP in 100 mM Tris-HCl pH 7.6, 20 mM $MgCl_2$, 20 mM NaCl and 200 µg ml^{-1} BSA), 6 µl of a decanucleotide solution (500 ng of random decamer oligodeoxynucleotides), 50 µCi α-^{32}P-dATP, 5 units of the Klenow fragment of DNA polymerase I were mixed with the DNA template and incubated at 37°C for 30 minutes. The radioactively-labelled DNA was separated from the unincorporated dNTPs by gel filtration through a Sephadex G-50 column (Pharmacia).

2.5.11.7 Hybridization of nucleic acids

The Zeta-probe membranes from Southern, northern or alkali-blots were processed in a Hybaid mini-system. Each membrane was soaked in 2x SSC buffer and transferred to a hybridization solution containing 293 mM Na_2HPo_4, 8% SDS, 1 mM EDTA. Each membrane was incubated in the hybridization solution at 65°C for 30-60 minutes. The solution was renewed and re-equilibrated to 65°C before the denatured DNA probe

(heated at 95°C for 5 minutes) was added to the hybridization solution. Hybridization was allowed to proceed overnight at 65°C.

The membranes were washed with two 5 minute rinses of 2x SSC and 0.1% SDS at room temperature, followed by two 15 minute washes in 0.1x SSC and 0.1% SDS at 65°C. The filters were blotted dry and covered with cling-wrap. The presence of radioactively-labelled bands was detected by autoradiography. The washed membranes were placed in a film cassette with either a X-OMAT or BioMax film (Kodak) and an appropriate intensifying screen. Cassettes were placed at -70°C for a period ranging from 4 to 48 hours before the films were developed and fixed.

Chapter 3

Expression of the ToLCV *c4* gene and a *c4 f-s* mutant in transformed plants,with analysis of theoretical characteristics of the putative translation products and comparison with homologues from other geminiviruses

3.1 Introduction

The limited studies of the C4 or AC4 ORF of begomoviruses have led to contradictory conclusions on its function in different viruses and host plants (Elmer *et al.*, 1988; Etessami *et al.*, 1991; Jupin *et al.*, 1994; Rigden *et al.*, 1994; Sung and Coutts, 1995; Hoogstraten *et al.*, 1996; Pooma and Petty, 1996). Structurally, the C4 or AC4 ORF of monopartite and bipartite viruses overlap the essential coding region C1 or AC1 (Figure 2). Single-point mutations that introduce a termination codon or destroy the initiator AUG codon of the C4 ORF without affecting the amino acids encoded by the C1 ORF have been used to assess the function of the C4 ORF of begomoviruses. This approach proved to be somewhat limited by occurrences of uncontrolled reversion of the introduced mutation back to the wild-type state which have been noted in some studies (Stanley and Latham, 1992; Jupin *et al.*, 1994; Rigden *et al.*, 1994).

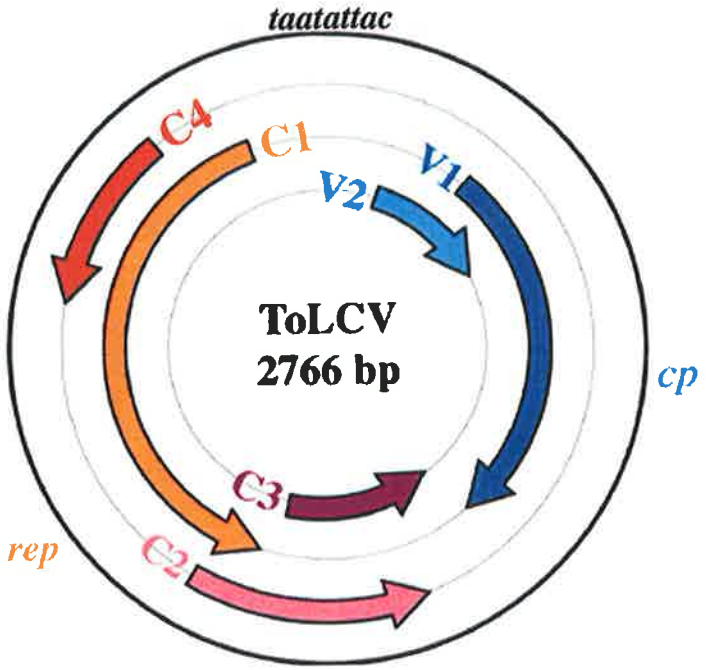
The experimental work described in this chapter overcomes this limitation by transforming plants with the ectopic *c4* gene. The previously published work by Rigden et al. (1994) with a C4 mutant version of ToLCV, like similar studies on the C4 ORF of BCTV by Stanley *et al.,* (1992), suggested that the C4 ORF product appeared to be involved with the development of disease symptoms in virus-infected plants. The primary aim of the following work was to test this hypothesis further by examining the phenotypic response of transformed tobacco and tomato plants expressing the ectopic *c4* gene. The secondary aim was to determine if the cause and effects were due to transcriptional expression of the *c4* transgene or mediated by the translation product of the C4 ORF.

3.2 Experimental procedure

3.2.1 Cloning the *c4* and *c4 f-s* genes

3.2.1.1 Sub-cloning the *c4* gene into pBluescript SK$^+$ (pBS*c4*)

The position of the C4 ORF within the genome of ToLCV is shown in Figure 19 (Dry *et al.*, 1993). A DNA fragment encompassing the entire C4 ORF was amplified from a full-length genomic clone using primers (nucleotides 2466 to 2449 and 2152 to 2172) incorporating *Hind*III and *Bam*HI restriction sites at their respective 5' ends (sections **2.5.9.2** and **2.5.9.3**).



ORF	Nucleotide start-stop	Predicted protein M_r
V1	308 - 1075	29,713
V2	148 - 492	13,250
C1	2615 - 1530	41,197
C2	1627 - 1223	15, 304
C3	1479 - 1078	16,114
C4	2464 - 2159	11,410

Figure 19. The genome organisation of ToLCV (Dry *et al.*, 1993).

The DNA product from the polymerase chain reaction was digested with 10 units each of *Hind*III and *Bam*HI (**2.5.3**) and the 315-bp DNA fragment fractionated by electrophoresis was recovered from the 1.5% NuSieve-TBE gel (**2.5.3**). Following recovery of the DNA fragment and dephosphorylation (**2.5.4**), the 315-bp DNA fragment was ligated (**2.5.10**) into a *Hind*III- and *Bam*HI-cut pBluescript SK^+ vector. Subsequently, the ligated vector was electroporated into *E. coli* strain DH5α cells (**2.5.8.2**). The recombinant bacterial cells were spread onto 1.2% agar plates containing 100 μg ml^{-1} ampicillin, 25 μg ml^{-1}, IPTG and 40 μg ml^{-1} of X-gal and incubated at 37°C for 16 hours. Selected white colonies were used to inoculate 2 ml liquid LB medium including 100 μg ml^{-1} of ampicillin and incubated at 37°C for 16 hours (**2.5.1.1**). The isolated plasmids from the cultures (**2.5.2.1**) were digested with 10 units each of *Hind*III and *Bam*HI restriction enzymes (**2.5.3**). The restricted plasmid preparations were fractionated by electrophoresis in a 1.5% agarose-TBE gel (**2.5.3**) to confirm the presence of the predicted 315-bp DNA fragment.

Selected colonies containing pBS*c4* were used to individually inoculate each 5ml liquid LB medium and cultured overnight at 37°C (**2.5.1.1**). The plasmids were isolated from the bacterial cells using the rapid boiling method (**2.5.2.1**), incubated with 0.2 mg ml^{-1} of ribonuclease A for 20 minutes at 37°C prior to extractions with phenol-chloroform, chloroform-isoamyl alcohol and precipitation with ethanol (**2.5.7**). The integrity of the C4 ORF was confirmed by sequencing (**2.5.10**).

3.2.1.2 The frame-shift version of the *c4* gene (*c4 f-s*)

A frame-shift version of the C4 ORF was prepared from 3 μg of pBS*c4*. The unique *Csp45*I site within the C4 ORF insert of pBS*c4* was digested with 20 units of *Csp45*I for 1 hour at 37°C and a 5μl aliquot fractionated by electrophoresis in a 0.8% agarose-TBE

gel (**2.5.3**), to confirm the presence of a 3200-bp band of restricted pBS*c4*. The remainder of the digest was purified by extraction with phenol-chloroform, followed by further extraction with chloroform-isoamyl alcohol and precipitated with 2 volumes of ethanol (**2.5.7**). The pellet was resuspended in 16 µl of sterile water and end-filled using T4 DNA polymerase (**2.5.5**) and blunt-end ligated (**2.5.6**) to produce pBS*c4 f-s*. The re-ligated plasmid was electroporated into *E. coli* strain DH5α cells (**2.5.8.2**) and spread onto 1.2% agar plates containing ampicillin, IPTG and X-gal and incubated at 37°C overnight (**2.5.1.1**).

Twenty-four white colonies were selected to separately produce overnight cultures in a LB medium containing ampicillin (**2.5.1.1**). The isolated plasmids (**2.5.2.1**) from 24 cultures were separately tested for resistance to restriction with 20 units of *Csp45*I and a sub-set of 12 were further tested for restriction with 10 units of *Hind*III (**2.5.3**). The digested plasmid DNA was fractionated by electrophoresis in 0.8% agarose-TBE gels (**2.5.3**). Four bacterial colonies containing the putative pBS*c4 f-s* were each used to inoculate 5ml of liquid LB medium plus ampicillin and cultured overnight at 37°C (**2.5.1.1**). The plasmids were isolated from the bacterial cells (**2.5.2.1**), and incubated with 0.2 mg ml^{-1} of ribonuclease A for 20 minutes at 37°C prior to extraction with phenol-chloroform, followed by extraction with chloroform-isoamyl alcohol and precipitation with ethanol (**2.5.7**). Each dried pellet was resuspended in 25 µl of sterile water and concentration of DNA determined by UV spectrophotometry before sequencing with a Sequenase DNA Sequencing Kit (**2.5.10**) to confirm the insertion of 2 bases after amino acid 14 of the C4 ORF.

3.2.1.3 Sub-cloning *c4* and *c4 f-s* into pJIT163 (Figure 20).

Selected DNA templates of pBS*c4* and pBS*c4 f-s* were digested with 10 units each of *Hind*III and *Bam*HI for 1.5 hours at 37°C prior to fractionation by electrophoresis in 1.5% NuSieve-TBE gel (**2.5.3**). The recovered 315-bp DNA of *c4* and 317-bp DNA of *c4 f-s* were each ligated (**2.5.6**) into *Hind*III-*Bam*HI-cut (**2.5.3**) and dephosphorylated (**2.5.4**) vector pJIT163 (Guerineau *et al.*, 1992). Each ligation reaction was electroporated into *E. coli* strain DH5α cells (**2.5.8.2**), spread onto 1.2% agar plates containing ampicillin, IPTG and X-gal and incubated at 37°C overnight (**2.5.1.1**).

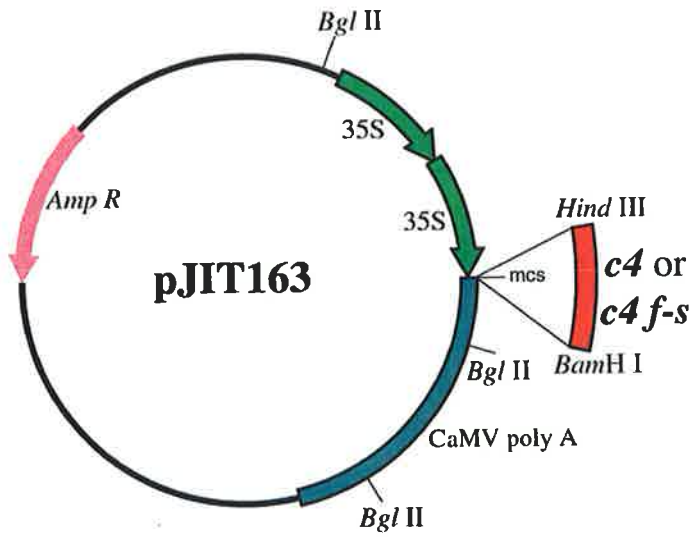


Figure 20. The sub-cloning strategy used for ligating *c4* and *c4 f-s* inserts into the *Hind*III-/*Bam*HI-cut pJIT163 plasmid.

Selected colonies were each used to inoculate 2ml of liquid LB medium plus ampicillin and cultured overnight at 37°C (**2.5.1.1**). The plasmids isolated from the cultures (**2.5.2.1**) were digested with 10 units of each of *Hind*III and *Bam*HI for 1 hour at 37°C before fractionation by electrophoresis in a 0.8% agarose-TBE gel to confirm the release of the 309-311-bp inserts. The remainder of a selected plasmid preparation of each of pJIT163-*c4* and pJIT163-*c4 f-s* were each digested with 20 units of *Bgl*II for 2 hours at

37°C and fractionated by electrophoresis in a 1.5% NuSieve-TBE gel (**2.5.3**). The released 1300-bp DNA fragments containing a tandem cauliflower mosaic virus (CaMV) 35S promoter, C4 ORF or C4 f-s ORF, and CaMV 35S terminator were recovered from the gel and stored at -20°C.

3.2.1.4 Sub-cloning 35*S-c4* and 35*S-c4 f-s* into pBin19 (Figure 21).

The thawed 1300-bp DNA fragments were ligated (**2.5.6**) into *Bam*HI-cut (**2.5.3**), dephosphorylated (**2.5.4**) pBin19 (Bevan, 1984) and electroporated into *E. coli* strain DH5α cells (**2.5.8.2**). The electroporated cells were spread on plates containing 1.2% Bacto-agar, 50 μg ml^{-1} of kanamycin, 25 μg ml^{-1} of IPTG, 40 μg ml^{-1} of X-gal and incubated at 37°C overnight. A selected white colony was used to inoculate 2.5ml of liquid LB medium containing 50 μg ml^{-1} of kanamycin and cultured at 37°C for 16 hours (**2.5.1.1**).

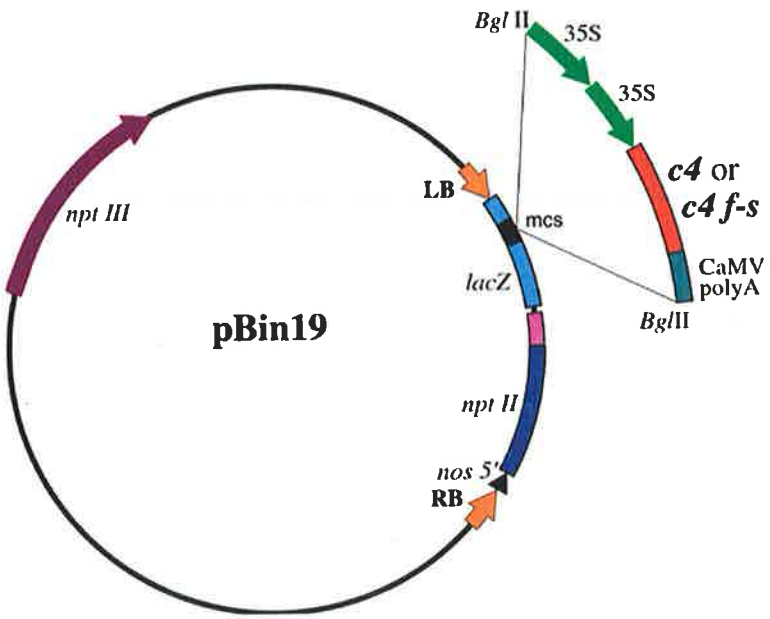


Figure 21. Sub-cloning strategy for ligating cassettes containing tandem 35S promoter , *c4* or *c4 f-s*,genes, and CaMV terminator into *Bam*HI-cut pBin19 plasmid.

An 8µl aliquot of a plasmid preparation (**2.5.2.1**) was digested with 20 units of *Hind*III and fractionated in a 1.2% agarose-TBE gel (**2.5.3**) to check the size of the restricted product. Additionally, another aliquot of the plasmid preparation was digested with 20 units of *Bgl*II and fractionated in a 0.8% agarose-TBE gel (**2.5.3**) to confirm the release of a 1300-bp DNA fragment. A further 1 µl of the selected plasmid preparation was used to electroporate pBin*c4* and pBin*c4 f-s* into *Agrobacterium tumefaciens* strain LBA4404 (**2.5.8.2**). The electroporated cells were spread onto plates containing 1.2% Bacto-agar, kanamycin (50 µg ml^{-1}), rifampicin (25 µg ml^{-1}) and incubated at 28°C for 48 hours. Selected colonies were used to each inoculate liquid LB medium containing kanamycin plus rifampicin and cultured overnight at 28°C (**2.5.1.2**). The isolated plasmids (**2.5.2.1**) were digested with 20 units of *Hind*III (**2.5.3**) and fractionated by electrophoresis in a 1.2% agarose-TBE gel to yield the predicted 700-bp fragments. Glycerol-stocks of *A. tumefaciens* strain LBA4404 containing either pBin*c4* (35*S-c4*) or pBin*c4 f-s* (35*S-c4 f-s*) were prepared from single selected bacterial colonies (**2.5.1.3**).

3.2.2 Production of transgenic plants

3.2.2.1 Transgenic tobacco

Tobacco tissue (*Nicotiana tabacum* cv. Samsun) was transformed with either 35*S-c4* or 35*S-c4 f-s* using essentially the method of Horsch et al. (1985). Detached leaves from plants maintained *in vitro* (**2.4**) were cut into pieces approximately 15 x 10mm and submerged for 3 minutes in a 10-fold dilution of an overnight culture of *A. tumefaciens* strain LBA4404 (4-6 O.D. 600nm) harbouring either the pBin*c4* or pBin*c4 f-s* plasmids (**2.5.1.2**). The leaf-pieces were drained, blotted dry and placed upside-down on the surface of petri-dishes containing a non-selection medium of 0.43% Murashige and Skoog medium, 3% sucrose, 0.1% Gamborg's vitamins, BA plus NAA, and 0.7% Bacto-agar (**2.4**). The leaf pieces were incubated for 2 days in the dark at 25-27°C prior to transfer to new petri dishes containing a selection medium of 0.43% Murashige and

Skoog medium, 3% sucrose, 0.1% Gamborg's vitamins, BA plus NAA, kanamycin (250 μg ml^{-1}), cefotaxime (500 μg ml^{-1}) and 0.7% Bacto-agar. These cultures were incubated at 25-27°C under artificial light (150 μMoles s^{-1} m^{-1}) for 16 h per day and sub-cultured monthly. The emerged shoots were sub-cultured into sterile plastic 110 x 60mm diameter vials containing 0.43% Murashige and Skoog medium, 3% sucrose, 0.1% Gamborg's vitamins, kanamycin and cefotaxime (**2.4**). The rooted plantlets were potted into plastic containers and maintained in a PH2 standard glasshouse compartment (**2.3**).

3.2.2.2 Transgenic tomato plants

Tomato explants (*Lycopersicon esculentum* cv. Ailsa Craig) were transformed with *A. tumefaciens* strain LBA4404 harbouring pBin*c4* or pBin*c4 f-s* recombinant binary plasmids using some of the protocols from (Horsch *et al.*, 1985; Bird *et al.*, 1988; Hamza and Chupeau, 1993). Freshly sampled 10-15mm segments of stem and hypocotyl from *in vitro* stock cultures of tomato (**2.4**) were submerged for 3 minutes in a 10-fold dilution of an overnight culture of *A. tumefaciens* strain LBA4404 (4-6 O.D. 600nm) harbouring either the pBin*c4* or pBin*c4 f-s* plasmids (**2.5.1.2**). The segments were drained, blotted dry and placed on the surface of petri-dishes containing 0.43% Murashige and Skoog medium, 3% sucrose, 0.1% Gamborg's vitamins, IAA aspartic acid (0.5 μg ml^{-1}), zeatin riboside (0.5 μg ml^{-1}) and 0.8% Phytagel (**2.4**). The inoculated segments were incubated in the dark at 25-27°C for 2 days prior to their transfer to new petri dishes containing the previous medium supplemented with kanamycin (100 μg ml^{-1}) and carbenicillin (500 μg ml^{-1}). These cultures were incubated at 25-27°C under artificial light (150 μE s^{-1} m^{-1}) for 16 h per day and sub-cultured every two weeks. The emerged shoots were sub-cultured into sterile plastic vials containing 0.43% Murashige and Skoog medium, 3% sucrose, 0.1% Gamborg's vitamins, kanamycin and carbenicillin

(200 μg ml^{-1}). The rooted plantlets were potted into plastic containers and maintained in a PH2 standard glasshouse compartment (**2.3**).

3.2.3 Analysis of transgene expression

RNA extracted from young leaves of transgenic plants (**2.5.11.4**) was fractionated by electrophoresis in 1.5% agarose-MOPS-formaldehyde gels under denaturing conditions and blotted onto nylon membranes (**2.5.11.5**). These membranes were hybridised at 65°C for 16 hours with DNA synthesised from a DNA template of 1630 bp fragment of ToLCV-DNA spanning the complementary-genes as described in section **2.5.11.6.** The washed and dried nylon membranes were autoradiographed as described in section **2.5.11.7**.

3.2.4 Database searches and analysis of genetic sequences

A suite of software available through WebAngis (http://www.angis.org.au) and BioNavigator (http://bionav.ebioinformatics.com) was used for database searches and amino acid comparisons. BLASTP was used to search various databases for related sequences. GAP was used for pair-wise comparisons using default settings for proteins and ClustalW was used for multiple sequence analysis. The molecular evolution was estimated from a distance matrix generated by Protdist and processed through Fitch, or Kitsch or Neighbor protocols of the PHYLIP package (Felsenstein, 1989) accessed through BioNavigator by eBioinformatics Pty Ltd. TreeView, version 1.2 (http://taxonomy.zoology.gla.ac.uk) was used to display the phylogenic analyses.

SeqVu version 1.1 (The Garvan Institute of Medical Research, Darlingurst, NSW) was used as an alignment editor with additional options including a hydropathy display of multiple amino acid sequences. Also, PSORT (http://psort.nibb.ac.jp) was used to search for localisation signals in the C4 and AC4 sequences.

3.3 Results

3.3.1 Gene constructs

3.3.1.1 pBS*c4*

The coding region of the C4 ORF of ToLCV (Dry *et al.*, 1993), consists of 309 nucleotides with a predicted translation product of 102 amino acids (Figure 22).

```
2464 atg aga atg ggg agc ctc atc tcc acg tgc
       M   R   M   G   S   L   I   S   T   C
2434 tta tcc agt tcg aag gca agt tcc agt gca
       L   S   S   S   K   A   S   S   S   A
2404 aga atc aac gat tct tcg acc tgg tct ccc
       R   I   N   D   S   S   T   W   S   P
2374 cca cca ggt cag cac att tcc atc cga aca
       P   P   G   Q   H   I   S   I   R   T
2344 ttc agg gag cta aga gct cgt cag acg tca
       F   R   E   L   R   A   R   Q   T   S
2314 agt cct atc tgg aga agg acg gag aca ccc
       S   P   I   W   R   R   T   E   T   P
2284 tcg aat ggg gag agt ttc aga tcg atg gac
       S   N   G   E   S   F   R   S   M   D
2254 gat ctg caa gag ggg gac aac aat cag cca
       D   L   Q   E   G   D   N   N   Q   P
2224 atg acg ctt acg ccc agg cgc tta aca ctg
       M   T   L   T   P   R   R   L   T   L
2194 gaa gta agt cag agg ctc tta acg tcc tta
       E   V   S   Q   R   L   L   T   S   L
2164 ggg aat tag
       G   N   *
```

Figure 22. The nucleic acid and amino acid sequence of the *c4* gene (Dry *et al.*, 1993).

The integrity of the 309-bp C4 ORF cloned into pBluescript SK$^+$ was confirmed by DNA sequencing. The presence of the introduced *Hind*III and *Bam*HI restriction sites at the ends of the C4 ORF were demonstrated with the release of a 315-bp DNA fragment from pBS*c4* following digestion with both enzymes (Figure 23).

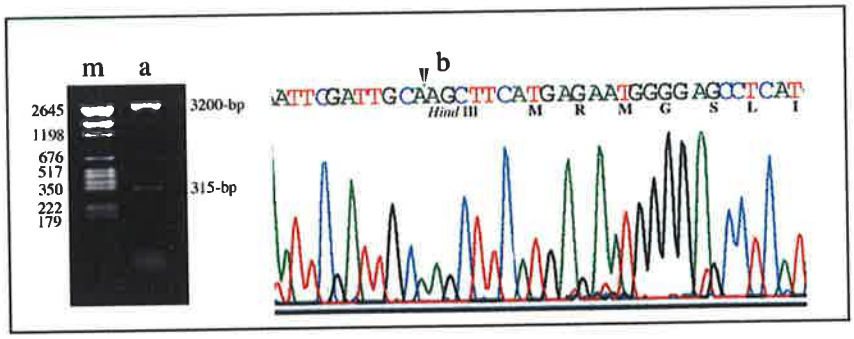


Figure 23. Release of the 315-bp DNA fragment containing the C4 ORF in a gel-fractionated digest of pBS*c4* with both *Hind*III and *Bam*HI enzymes (a). A section of DNA sequence of the cloned C4 ORF (b) confirming the location of the *Hind*III restriction site. Molecular markers (m) are shown for comparison.

3.3.1.2 pBS*c4 f-s*

The strategy used for creating a frame-shift mutation within the *c4* gene is depicted in Figure 24. The addition of a base at position 2425 (c) and 2424 (g) of the C4 ORF within pBS*c4* was confirmed through DNA sequencing. The insertion of 2 bases was designed to cause a frame-shift of the *c4* translation product. The putative translation product of the 311-bp *c4 f-s* gene (Figure 25) is predicted to be a polypeptide of 44 amino acids (aa) with the N-terminal group of 14 aa the same as in the native C4 protein.

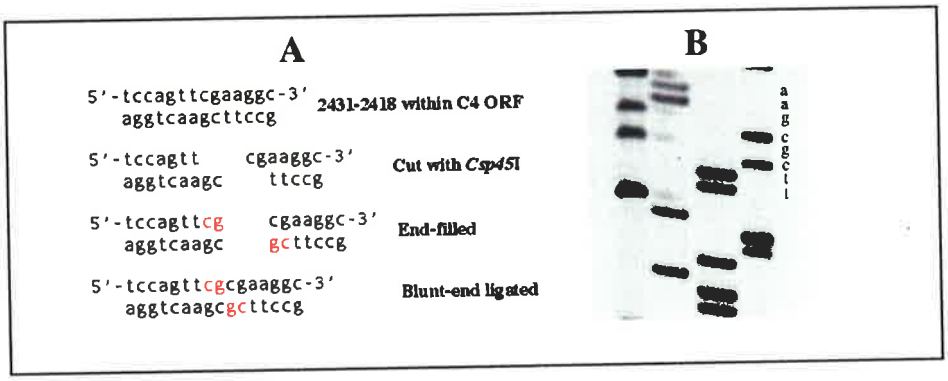


Figure 24. Strategy used for creating the frame-shift mutation within the *c4* gene (a). The insertion of 2 bases within pBS*c4 f-s* was confirmed by the ttcgcgaa sequence (b).

```
2464 atg aga atg ggg agc ctc atc tcc acg tgc
       M   R   M   G   S   L   I   S   T   C
2434 tta tcc agt tcg cga agg caa gtt cca gtg
       L   S   S   S   R   R   Q   V   P   V
2404 caa gaa tca acg att ctt cga cct ggt ctc
       Q   E   S   T   I   L   R   P   G   L
2374 ccc cac cag gtc agc aca ttt cca tcc gaa
       P   H   Q   V   S   T   F   P   S   E
2344 cat tca ggg agc taa gag ctc gtc aga cgt
       H   S   G   S   *
2314 caa gtc cta tct gga gaa gga cgg aga cac

2284 cct cga atg ggg aga gtt tca gat cga tgg

2254 acg atc tgc aag agg ggg aca aca atc agc

2224 caa tga cgc tta cgc cca ggc gct taa cac

2194 tgg aag taa gtc aga ggc tct taa cgt cct

2164 tag gga att ag
```

Figure 25. The nucleotide and amino acid sequence of the *c4 f-s* gene showing the insertion of cg and the change in the predicted amino acid sequence (blue).

3.3.1.3 Cassette of 35S-*c4* or *c4 f-s* gene-35S terminator

Following the ligation of *c4* and *c4 f-s* inserts into *Hind*III- and *Bam*HI-cut pJIT163 (see Figure 20), a 1300-bp cassette containing a tandem 35S promoter-*c4* or *c4 f-s* gene-35S terminator was released by separately digesting the pJIT163*c4* and pJIT163*c4 f-s* plasmids with *Bgl*II enzyme. An example of the fractionated digest of pJIT163*c4 f-s* is shown in Figure 26A. The purified 1300-bp DNA fragments were each ligated into the binary vector pBin19 and digested with *Hind*III to confirm the release of a predicted 700-bp DNA fragment indicating an intact insert within the pBin19 plasmid (Figure 26B).

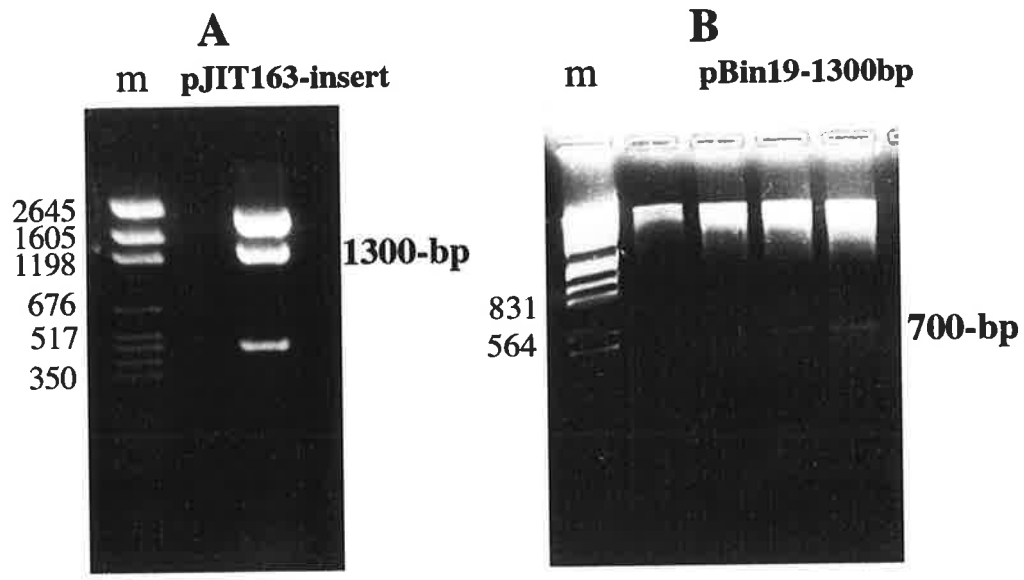


Figure 26. The fractionated digest of pJIT163-insert with *Bgl*II showing the release of the 1300-bp cassette containing the tandem 35S promoter-*c4* or *c4 f-s*-35S terminator (A). The fractionated digest of pBin19-1300-bp insert with *Hind*III to confirm the cassette within pBin19 (B). The size of fragments determined by comparison with a ladder of DNA markers (m).

3.3.2 Nature of transgenic plant phenotypes

3.3.2.1 Tobacco

Transgenic tobacco plants made with each gene construction were derived from cultures of 30 leaf pieces previously inoculated with *A. tumefaciens* strain LBA4404 containing either pBin*c4* or pBin*c4 f-s* plasmids. The first sign of callus development on the leaf pieces was observed 2 weeks after incubation with the recombinant bacterial culture. By the third week, the first shoots emerged on the kanamycin selection medium (Figure 27). These were harvested and transferred to fresh selection media without hormones. None of the cultures showed any signs of bacterial overgrowth.

Figure 27. A culture of tobacco leaf explants on a kanamycin-selection plate showing the development of callus and the emergence of shoots.

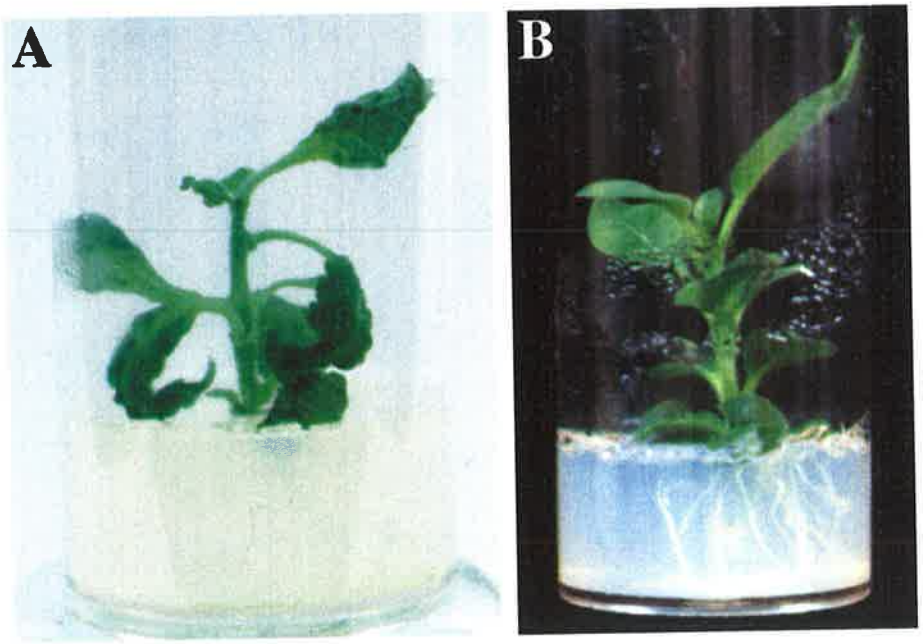


Figure 28. An abnormal (A) and phenotypically normal kanamycin-selected shoot (B) derived from *in vitro* cultures of 35S-*c4* and 35S-*c4 f-s,* respectively.

It was evident from an early stage that some of the kanamycin-resistant shoots derived from 35S-*c4* cultures were severely deformed and failed to develop roots. In contrast, all the kanamycin-resistant shoots harvested from cultures of 35S-*c4 f-s* appeared normal and readily formed roots. At least 50 selected shoots of each of the 35S-*c4* and 35S-*c4 f-s* constructs were maintained *in vitro* until roots developed (Figure 28). A population of thirty-six 35*S-c4* rooted plantlets were selected for further analysis. Of these, seventeen plantlets appeared normal, eighteen plantlets were moderately deformed and one plantlet severely malformed. These selected plants were transferred into containers of sand and peat moss (**2.3**) and maintained in a PH2 glasshouse for further analysis. Likewise, a similar number of plantlets regenerated from the 35*S-c4 f-s* cultures were transferred to the PH2 glasshouse for comparative studies with the 35*S-c4* derived plants.

Transgenic tobacco lines containing the 35S-*c4* construct displayed a range of foliar symptoms (Figures 30-33) including modified leaf shapes with varying degrees of rugosity, blistering, twisting, curling of leaf margins and the presence or absence of mosaic patterns. Some plants also showed growth with thickened stems, enations, zigzag growth pattern (Figures 30, 32) and tumorous growths on deformed flowers (Figure 33). In particular, the puckering, blistering and curling of leaves were common symptoms displayed by all abnormal plants derived from *35S-c4* cultures (Table 14). These leaf symptoms also resembled those induced by ToLCV in Samsun tobacco and *N. benthamiana* plants (Figure 29).

Figure 29. Typical foliar symptoms caused by infection with ToLCV in wild-type *N. tabacum* var. Samsun (A) and *N. benthamiana* (B).

Figure 30. Examples of foliar symptoms displayed by transgenic tobacco plants derived from 35*S-c4* cultures.

Table 14. Summary of foliar symptoms displayed by kanamycin-selected plants derived from 35*S*-*c4* and 35*S*-*c4*-*f*-*s* cultures.

Putative transgenic lines	Foliar symptoms
35*S*-*c4*-D2, E1, L3, P4, P5, Q1, Q3, R1, S5, T2, W1, Z1, α1, β3, β4, Δ1, Δ3 All 35*S*-*c4* *f*-*s* lines (40)	Symptomless growth
35*S*-*c4*-B3, P3, X5.	Moderately deformed growth. Narrower laminae, bubbled, blistered and curled with yellowish and/or dark-green areas of mosaic
35*S*-*c4*-A1, A2, B1, B2, C1, E5, G1, L1, L2, P1, U3, β1, β2, β5, β6.	Moderately deformed growth. Smaller laminae, bubbled, blistered, twisted with curling of leaf margins. Deformed flowers with enlarged calyx
35*S*-*c4*-K4	Severely deformed growth. Blistered, twisted and curled laminae with enations on thickened zig-zag shoots. Deformed and enlarged flowers with tumorous growths on the calyx

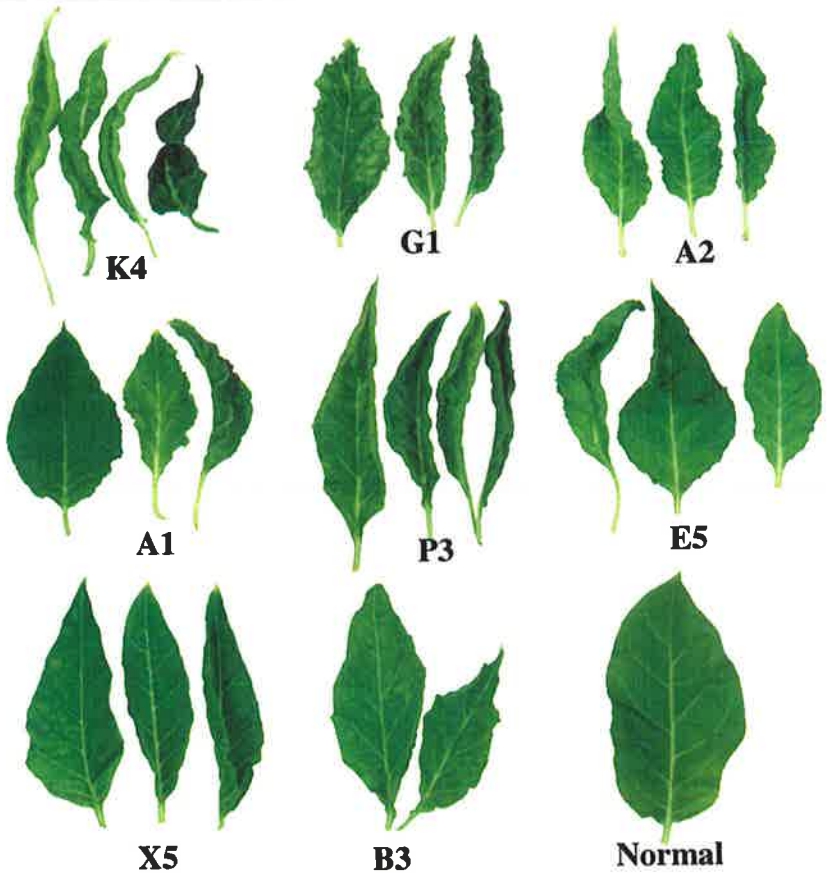


Figure 31. Representative leaf symptoms displayed by selected transgenic tobacco lines derived from the 35S-*c4* cultures.

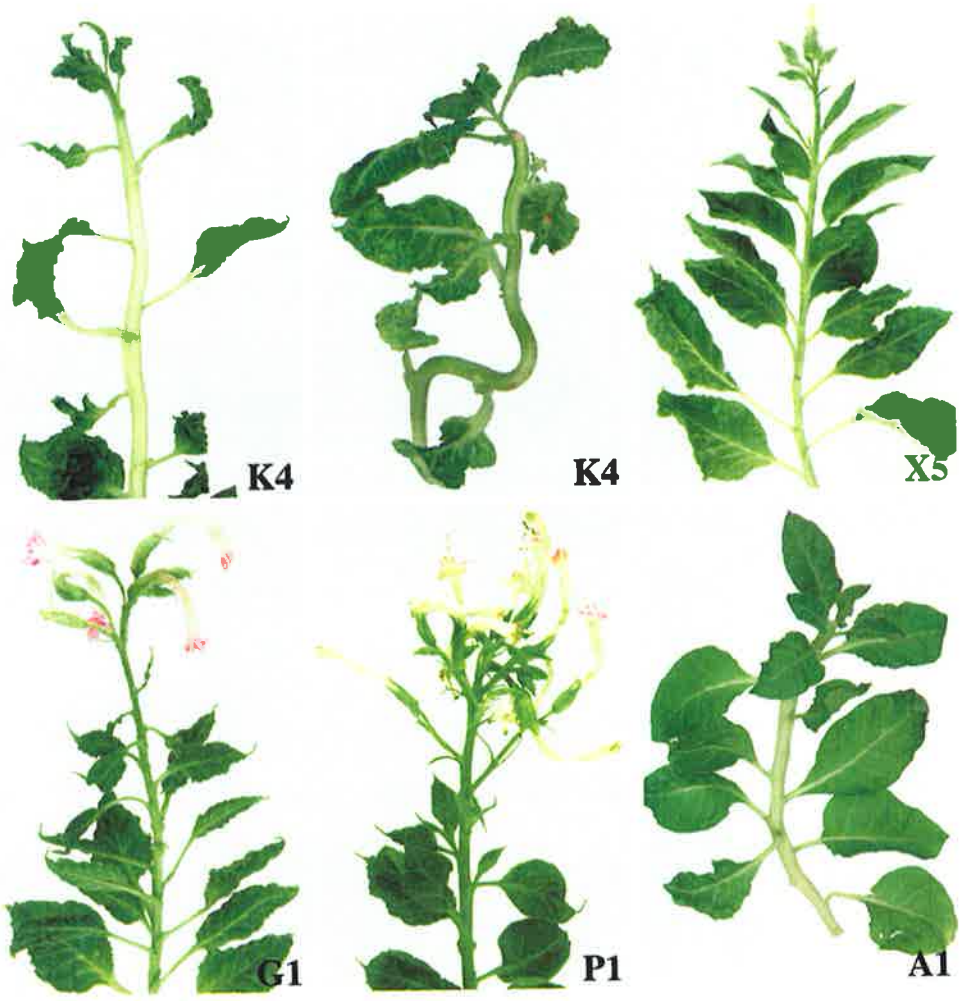


Figure 32. Representative shoots from selected *c4* transgenic plants displaying virus-like symptoms.

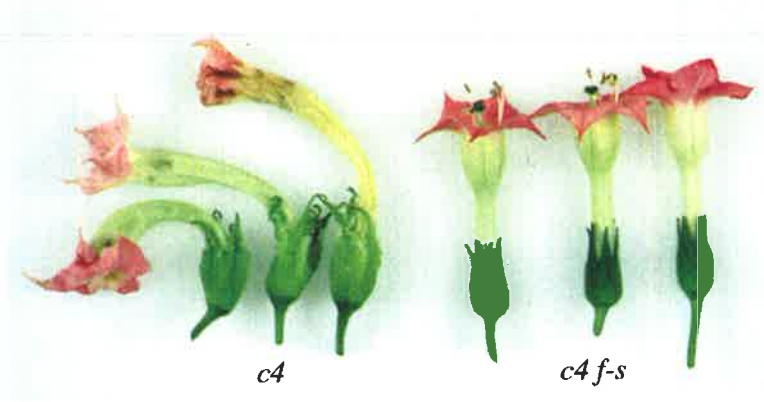


Figure 33. Abnormal flowers produced by *c4* transgenic tobacco lines compared with normal flowers from *c4 f-s* tobacco lines.

All kanamycin-resistant plants containing the 35S-*c4 f-s* construct were normal (Figure 34) and could not be distinguished from native *N. tabacum* var. Samsun plants.

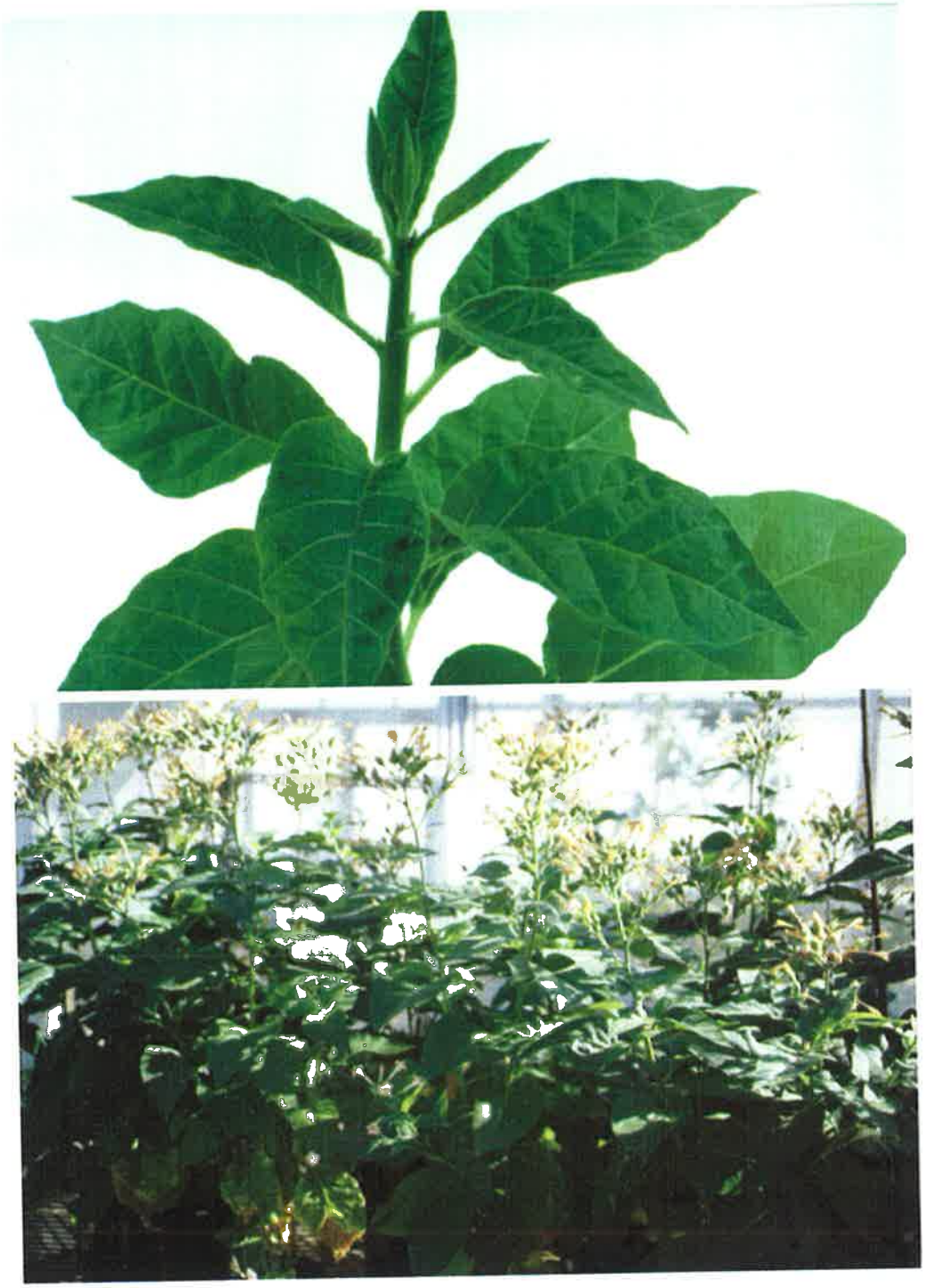


Figure 34. Examples of kanamycin-resistant plants regenerated from the 35S-*c4 f-s* cultures.

3.3.2.2 Tomato

Both the 35S-*c4* and 35S-*c4 f-s* constructs were used to produce a total of 30 kanamycin-resistant tomato lines. Approximately 50% of the 200 stem and hypocotyl explants regenerated shoots. Most of the shoots obtained were harvested from the stem segments and few shoots were obtained from the hypocotyl explants (Figure 35).

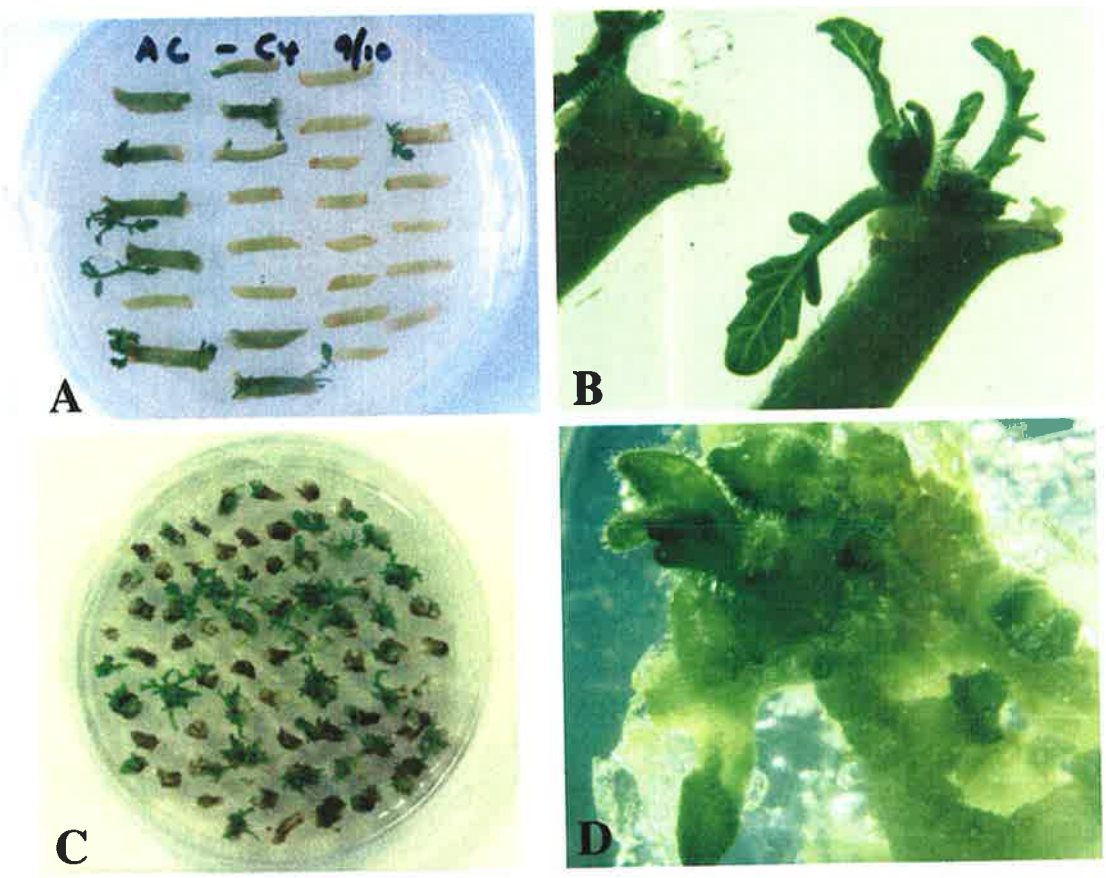


Figure 35. *In vitro* culture of kanamycin-selected tomato shoots.

All of the shoots regenerated with the 35S-*c4 f-s* construct developed into normal plantlets (Figure 36B). In contrast, the shoots harvested from the 35S-*c4* cultures were variable in phenotype. Some were rosellate and failed to develop any extended growth or roots, while others appeared normal or developed varying degrees of twisting and curling of the leaves (Figure 36A).

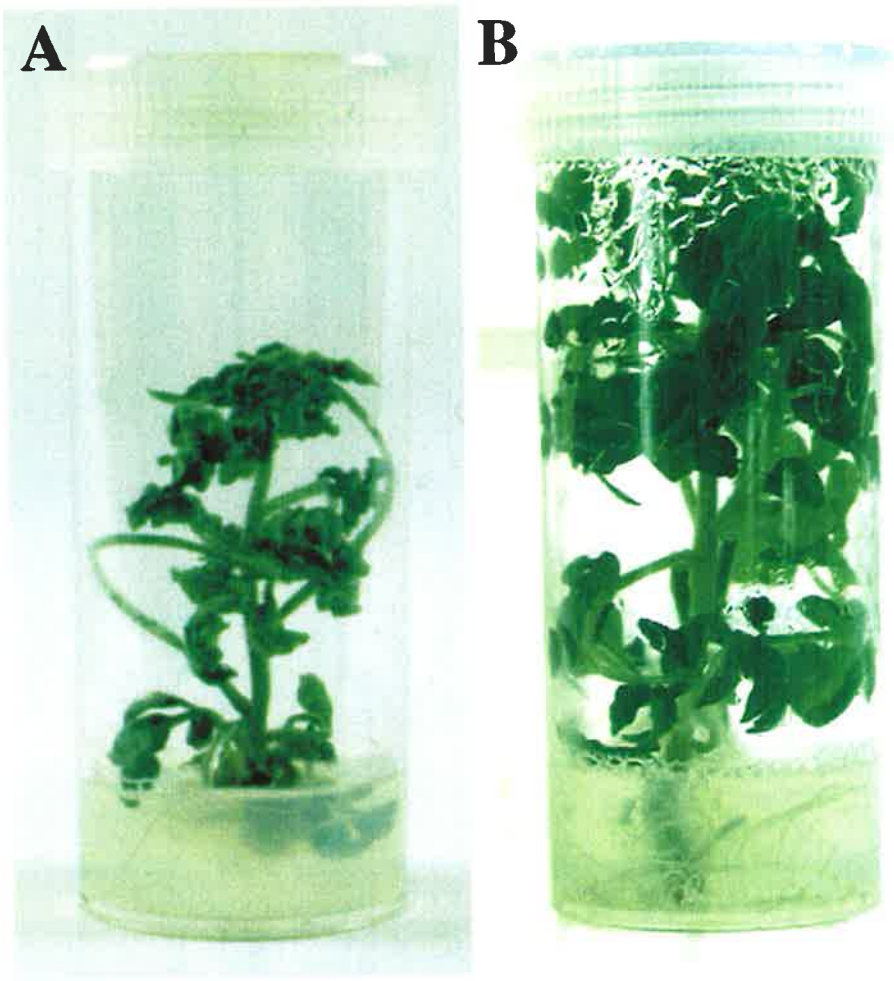


Figure 36. Examples of abnormal (A) and normal (B) kanamycin-selected tomato plantlets derived from 35*S-c4* and 35*S-c4 f-s* cultures, respectively.

All of the twenty-one kanamycin-resistant tomato lines derived from 35S-*c4 f-s* cultures that were transferred and grown in a PH2 glasshouse produced normal growth that could not be distinguished from the non-transformed Ailsa Craig plants. The surviving eight rooted plants derived from the 35S-*c4* cultures continued to develop abnormal growth when transferred and grown in the glasshouse. These abnormal phenotype plants were smaller than normal and displayed varying degrees of curled and twisted leaflets through to the most grotesque that additionally expressed enations, tumorous growths on the stems, petioles and flowers (Figures 37, 38, Table 15).

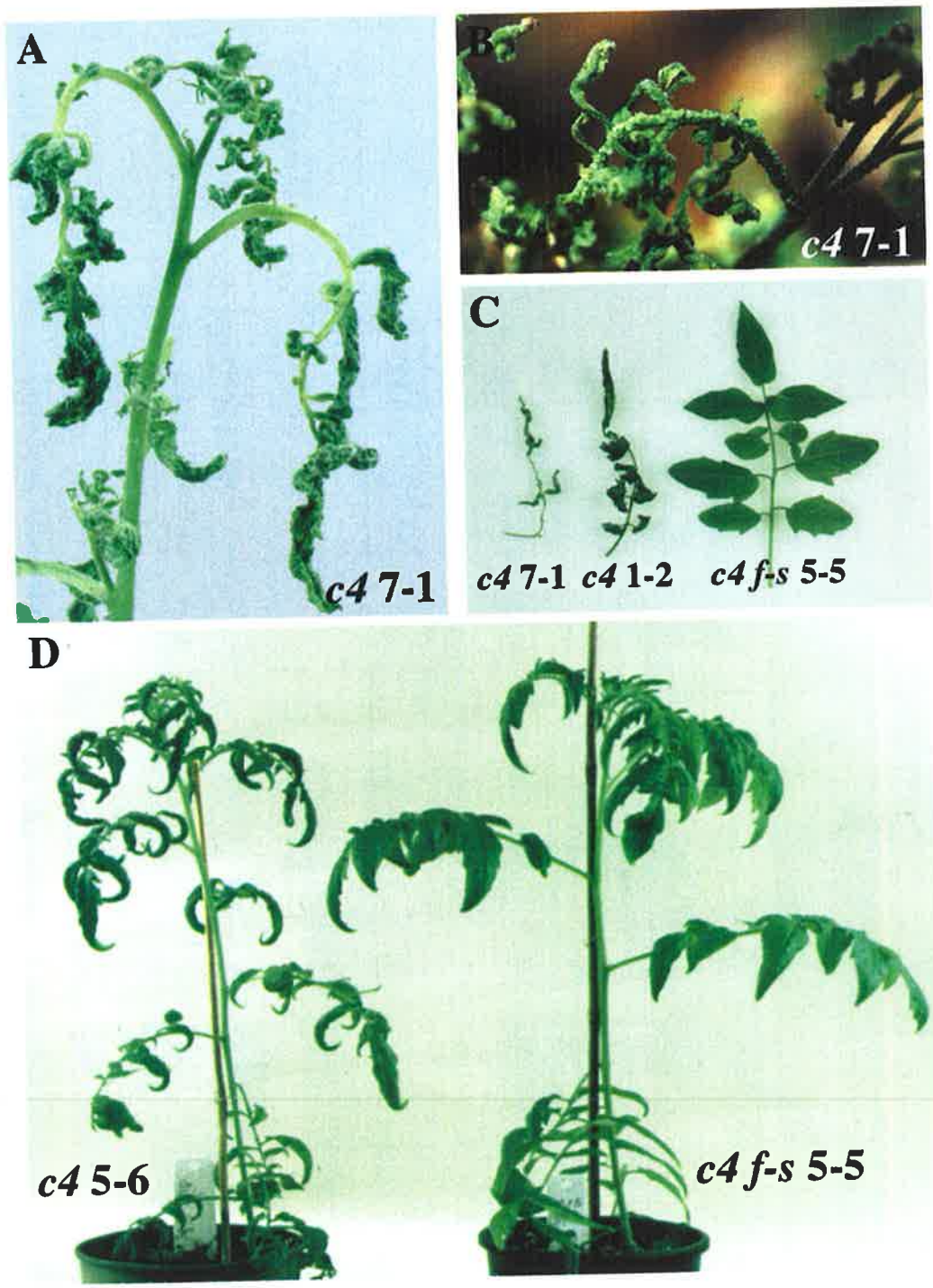


Figure 37. Abnormal foliar symptoms induced in transgenic tomato plants expressing the *c4* gene from ToLCV under the control of the CaMV 35S promoter showing varying degrees of deformed growth (A, B, C, D) including formation of enations and tumorous growth compared with selected transgenic 35S-*c4 f-s* lines (C, D).

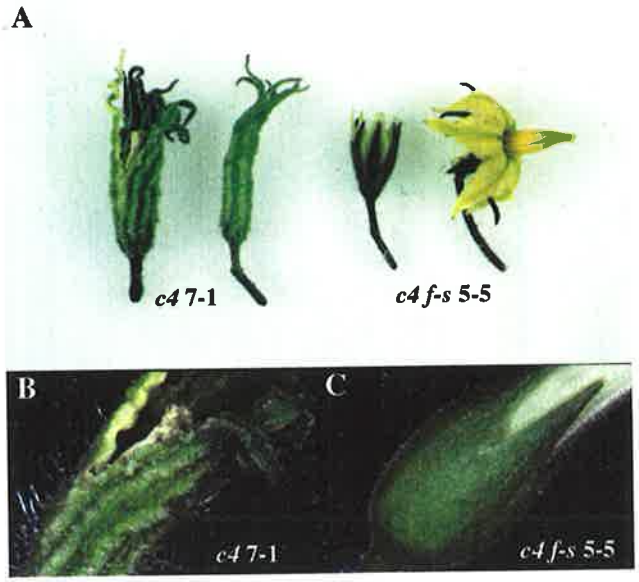


Figure 38. The inflorescences produced by the *c4* 1-7 transgenic tomato line (A) developed an abnormally elongated calyx, deformed sepals and longitudinal ribs of tumorous enations in constrast to normal flowers produced on all *c4 f-s* transgenic tomato lines. A close-up of a *c4* 1 elongated calyx (B) shows the formation of tumorous enations and enlarged trichomes compared with a normal tomato calyx (C).

Table 15. Examples of foliar symptoms displayed by kanamycin-selected plants derived from 35*S*-*c4* and 35*S*-*c4 f-s* cultures.

Putative transgenic tomato plants	Foliar symptoms
All 35*S*-*c4*-*f*-*s* plants	Symptomless
35*S*-*c4*-(2-1), (2-4), (1-6),	Mildly abnormal with slight downward curling of almost normal-sized leaflets
35*S*-*c4*-(1-1), (1-8), (5-6)	Moderately abnormal with pronounced downward curling of narrower and smaller leaflets
35*S*-*c4*-(1-2), (7-1)	Severely deformed and stunted leaflets, tumorous outgrowths on stems and petioles. Enlarged and deformed flower buds with tumorous enations

3.3.3 Expression of the *c4* and *c4 f-s* transgenes

3.3.3.1 Correlation of *c4* gene expression with abnormal plant phenotypes

The Northern-blot analysis of total RNA (**2.5.11.5** to **2.5.11.7**) extracted from kanamycin-selected transgenic 35S-*c4* tobacco lines is shown in Figure 39. The *c4* transcript was detected in all abnormal transgenic tobacco and tomato plants containing the 35S-*c4* construction. No significant expression of the *c4* transgene was observed in extracts of normal plants derived from 35S-*c4* cultures and no background reactions were observed with extracts of healthy plants despite the finding of geminivirus-like sequences in the genome of *N. tabacum* (Bejarano *et al.,* 1996; see Figure 52A and B). The level of transgene expression was found to generally increase in accordance with the observed degree of abnormality of the abnormal phenotypes. This analysis supports a close association between the level of *c4* transgene expression and the appearance of virus-like symptoms in the 35S-*c4* transgenic tobacco plants.

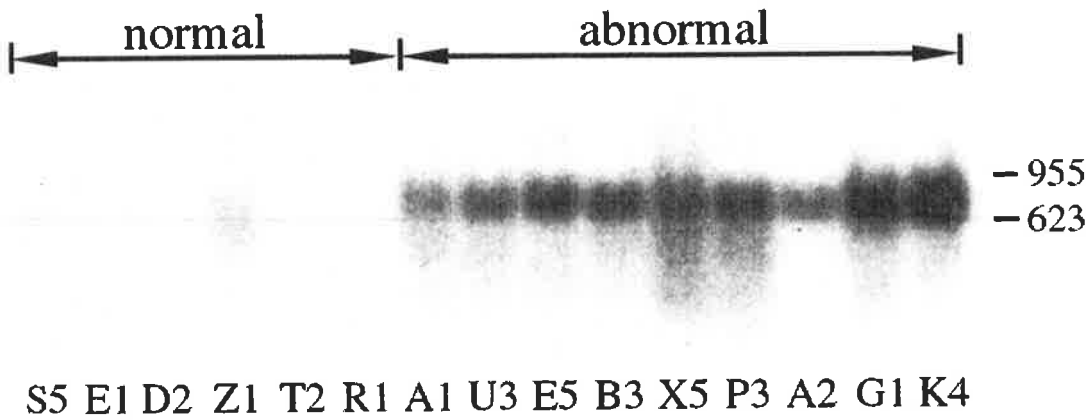


Figure 39. Analysis of transgene expression in selected 35S-*c4* transgenic tobacco plants. Total RNA (20 µg per lane) was loaded from left to right in order of severity of the virus-like symptoms. The position of relevant RNA markers (nucleotides) is also shown.

3.3.3.2 Comparison of *c4* and *c4 f-s* transgene expression

Northern-blot analysis of the total RNA extracted from kanamycin-selected transgenic 35S-*c4 f-s* tobacco lines showed that many of the kanamycin-resistant lines displaying a normal phenotype were expressing high levels of the *c4 f-s* transcript. Direct comparison of transgene expression from different abnormal 35S-*c4* transgenic lines with selected 35S-*c4 f-s* transgenic lines showed comparable levels of transcription (Figure 40). This comparison demonstrated that the induction of abnormal growth of 35S-*c4* transgenic plants resulted directly from the translation product of the *c4* gene instead of being caused by the level of RNA transcript expression *per se*.

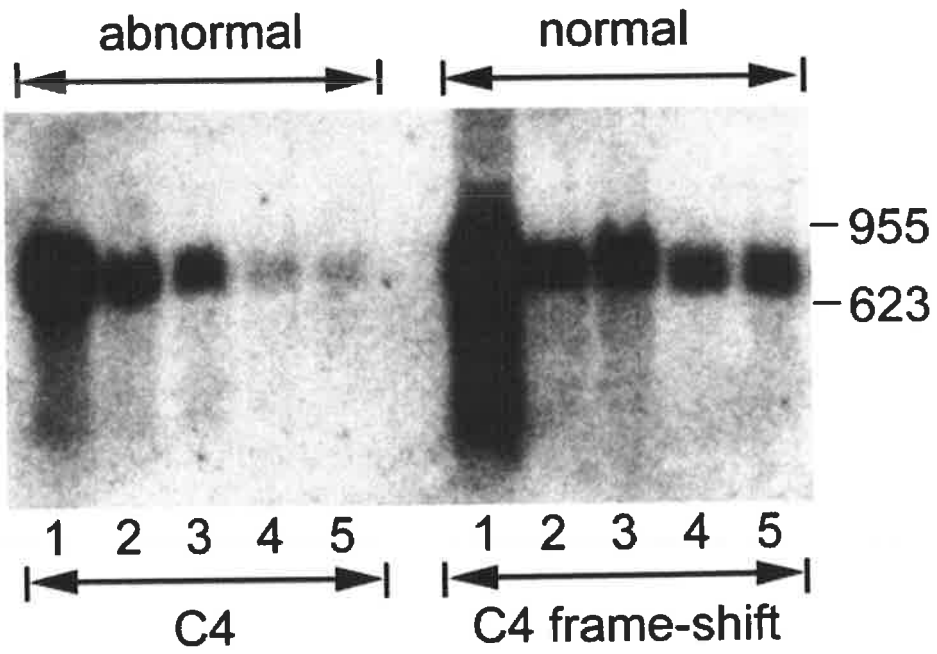


Figure 40. Comparison of the expression level of 35S-*c4* and 35S-*c4 f-s* transcripts in selected transgenic tobacco lines. RNA extracts from five individual abnormal 35S-*c4* plants were compared with examples of 35S-*c4 f-s* transgenic lines. The position of relevant RNA markers (nucleotides) is also shown.

3.3.3.3 Expression of the *c4* and *c4 f-s* transgenes in tomato

The northern-blot analysis of kanamycin-selected tomato plants derived from the transformed cultures of 35*S*-*c4* and 35*S*-*c4 f-s* shown in Figure 41 gave similar patterns of expression as shown in Figures 39 and 40. For instance, the level of transcript obtained from the *c4* transgene (Figure 41A) corresponded to the degree of abnormality displayed by the abnormal phenotypes as shown in the comparison of severely abnormal phenotypes (lines 1-2 and 7-1) with moderately abnormal (line 1-1) and mildly abnormal plants (line 2-1). The northern-blot hybridisation on a random selection of regenerated tomato lines from the 35*S c4 f-s* cultures showed that only two lines tested out of a selection of ten gave expression of the *c4 f-s* transgene (Figure 41B). The normal phenotype produced by the 5-5 line expressed the *c4 f-s* transgene to a level exceeding that expressed by the severely abnormal 1-2 and 7-1 lines derived from the 35*S*-*c4* cultures. The pattern of expression obtained from the 6-1 line was unusual in that a major and minor band of viral RNA appeared either side of the *c4 f-s* marker (+). All of the other lines analysed failed to express the *c4 f-s* transcript.

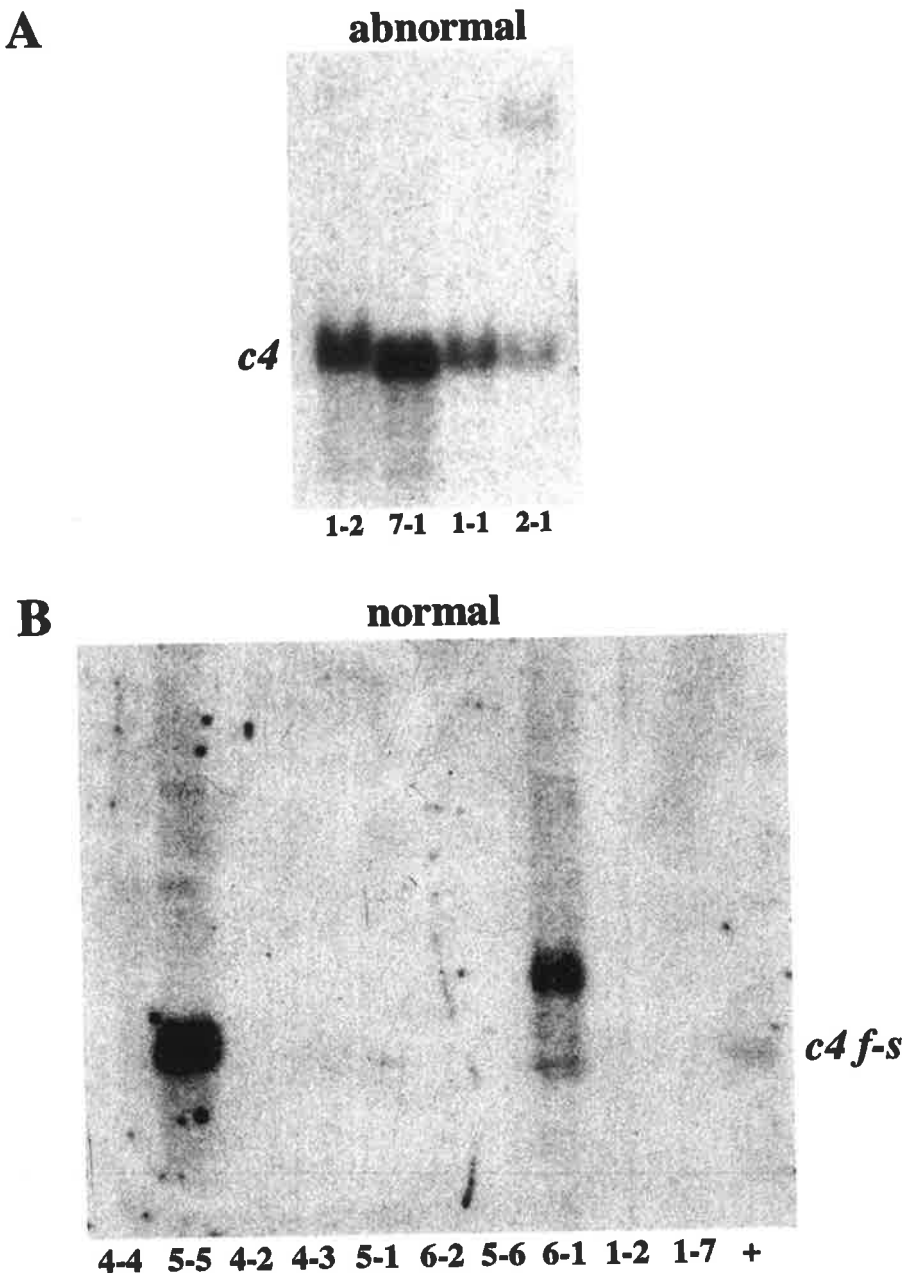


Figure 41. The expression of the *c4* transgene (A) in transformed tomato plants that developed severely abnormal foliage (lines 1-2 and 7-1), moderately abnormal foliage (line 1-1) and mildly abnormal foliage compared with the expression of the *c4 f-s* transgene (B) in a random selection of regenerated tomato lines that appeared normal. An extract of a transformed 35*S c4 f-s* tobacco plant (+) was used as an internal marker.

3.3.4 The heritability and stability of the *c4* gene

The parental abnormal 35S-*c4* transgenic tobacco plants were maintained under glasshouse conditions for 2 years to observe stability of the induced phenotypes. During this period, all transgenic plants were inspected and cut back after each flush of flowering. All phenotypes continued to produce systemic virus-like symptoms although 10 of the abnormal phenotypes (A1, A2, B1, B2, B4, E5, G1, L1, L2, U3) were observed to produce an occasional normal shoot among the bulk of abnormal foliage (Figure 42A, B).

RNA was extracted from two pairs of normal and abnormal leaf tissues sampled from different transgenic lines. Northern-blot analysis of the extracts revealed that the *c4* transgene was undetectable in leaves of all normal shoots developing on abnormal phenotype plants (Figure 42C). The absence of *c4* transcript in the normal growth of different transgenic lines further supported the link between expression of the *c4* transgene with the virus-like symptoms and suggested that gene silencing or rejection of the transgene occurred within some lateral meristem cells of abnormal transgenic plants. None of the affected plants completely reverted to normal and this gene-silencing trait was lost from the abnormal phenotype plants when the normalised shoots were removed.

Germination of self-pollinated seedlings from T_0 transgenic lines on kanamycin plates (Figure 43) indicated that the lines had different numbers of gene insertions (Table 16). Several of the 35S-*c4* lines showed 3:1 segregation whereas some of the other lines had higher ratios of kanamycin resistant plants suggesting that there were multiple inserts of the gene at different loci.

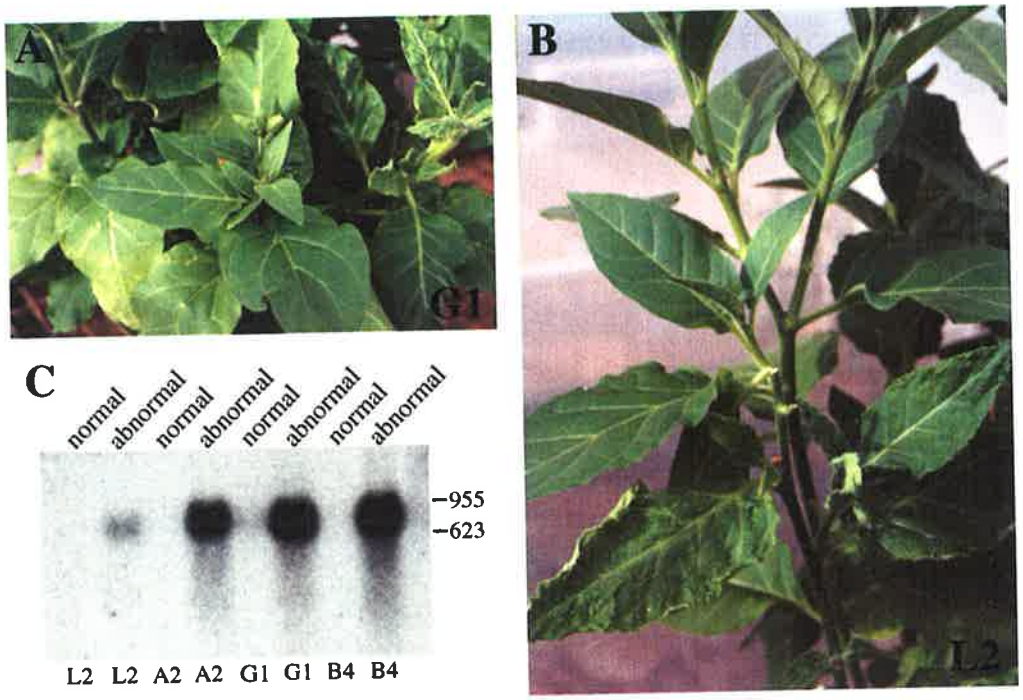


Figure 42. Examples of normal growth that emerged from lateral meristems of abnormal transgenic tobacco plants (A and B). RNA extracted from normal and abnormal tissues was compared to determine the expression of the *c4* gene transcript. The position of relevant RNA markers (nucleotides) is also shown.

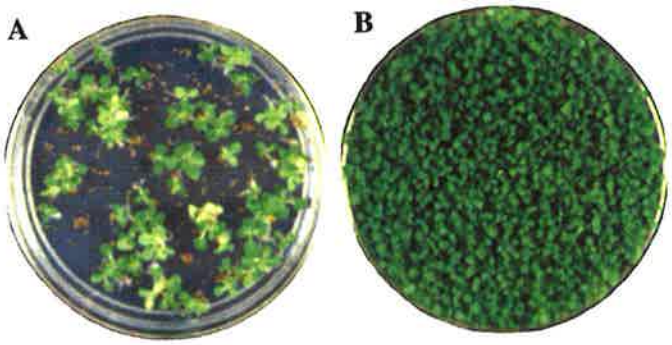


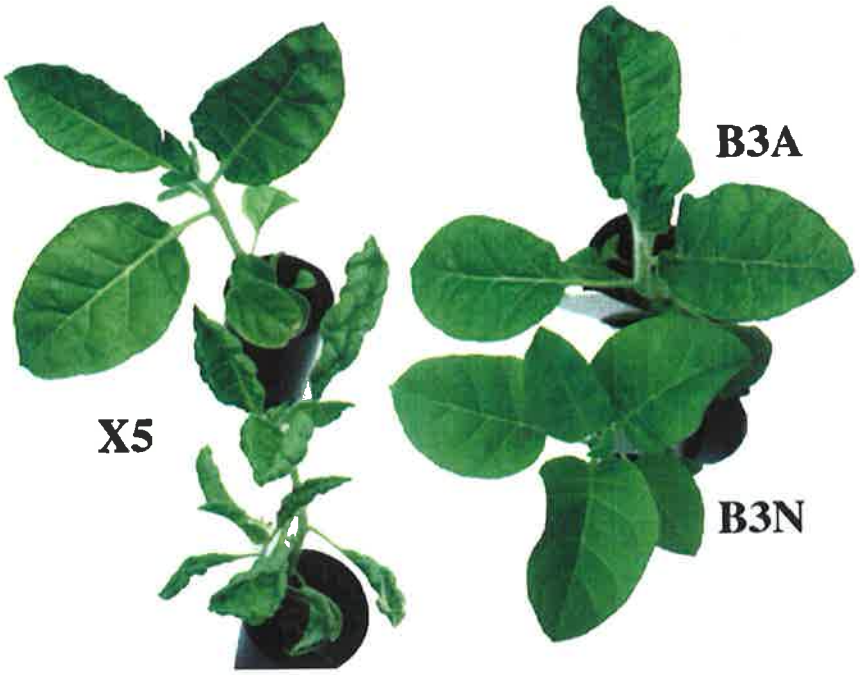
Figure 43. The germination of T_1 generation seed from 35S-*c4* transgenic tobacco on media containing kanamycin to select transformed plants. A seedling population showing 3:1 segregation of kanamycin-resistant to kanamycin-sensitive seedlings (A) and a population of kanamycin-resistant seedlings from a transgenic line with multiple inserts of the *kan* gene (B).

Table 16. The number of T_1 seedlings from 35S-*c4* lines that were resistant (Kan^+) or sensitive (Kan^-) to kanamycin selection.

T_1 35S-*c4* line	Number of plants $Kan^+ : Kan^-$	Segregation
B1	183 : 14	13 : 1
B2	123 : 30	4.1 : 1
B3	107 : 29	3.7 : 1
B4	70 : 33	2 : 1
D1	117 : 28	4.2 : 1
D2	136 ; 46	2.95 : 1
D3	106 : 28	3.8 : 1
E1	87 : 35	2.5 : 1
G1	40 : 9	4.4 : 1
K4	94 : 26	3.6 : 1
L1	82 : 24	3 : 1
L2	131 : 43	3 : 1
L3	99 : 34	2.9 : 1
P1	114 : 23	4.95 : 1
P3	113 : 9	12.5 : 1
Q1	109 : 31	3.5 : 1
Q3	122 : 10	12.2 : 1
R1	100 : 0	100%
S5	94 : 31	3 : 1
W1	92 : 30	3 : 1
X5	105 : 35	3 : 1
Z1	61 : 25	2.4 : 1

A proportion of the T_1 seedlings of each transgenic line displayed the same virus-like symptoms as the parental 35S-*c4* source indicating stable integration of the *c4* gene insert and heritability of the virus-like symptoms (Figure 44). Several of the 35*S-c4* transgenic lines showed a segregation of phenotypes in the T_1 progeny. For example, the X5 line showed a 2:1 segregation of populations with severely deformed phenotypes and those with moderately deformed growth (Figure 44A). The analysis of transgene expression in the representative plants showed a major *c4* mRNA species (Figure 44B) corresponding to that produced in the parental T_0 transgenic line, as shown by X5 in Figure 39. The second example of the B3 line showed segregation of normal and moderately abnormal phenotypes in the T_1 seedlings (Figure 44A). By comparison, the level of *c4* mRNA was considerably lower in extracts of both the mildly abnormal (B3A) and normal (B3N) plants (Figure 44B) than that originally obtained from the B3 parental line (Figure 39). The presence of multiple banding of higher molecular weight RNA species in the extracts of the B3 T_1 progeny appear to indicate the lack of complete RNA processing, as a single RNA species was produced in the extracts of the parental line.

A



B

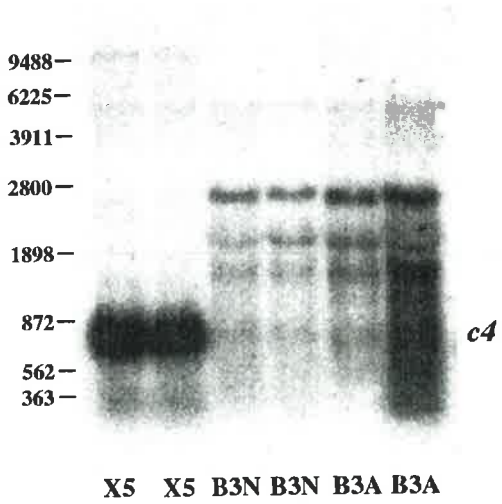


Figure 44. Examples of segregated T_1 35S-*c4* phenotypes obtained following selection of seedlings germinated on kanamycin plates showing heritability of the virus-like symptoms (A) and expression of transgene RNA (B). The position of relevant RNA markers (nucleotides) is also shown.

3.3.5 Predicted characteristics of the C4 protein and a hypothetical C4 f-s product

A comparison of the theoretical biochemical properties of the C4 protein and a hypothetical C4 f-s translation product are shown in Figure 45. The estimated molecular weights of the C4 protein and the putative C4 f-s product are predicted to be 11409 and 4782 Daltons, respectively. The amino acid composition of the polypeptides are somewhat similar (Figures 45A, 45C and 45D), allowing for the fact that the C4 f-s product would be only 43% of the length of the C4 polypeptide. On the other hand, the predicted isoelectric point for the C4 polypeptide is estimated to be 11.5 compared with 10.61 for the C4 f-s protein. Assuming that the *c4 f-s* sequence translates into a polypeptide, these theoretical differences may indicate differing biochemical characteristics between the translation products (Figure 45B).

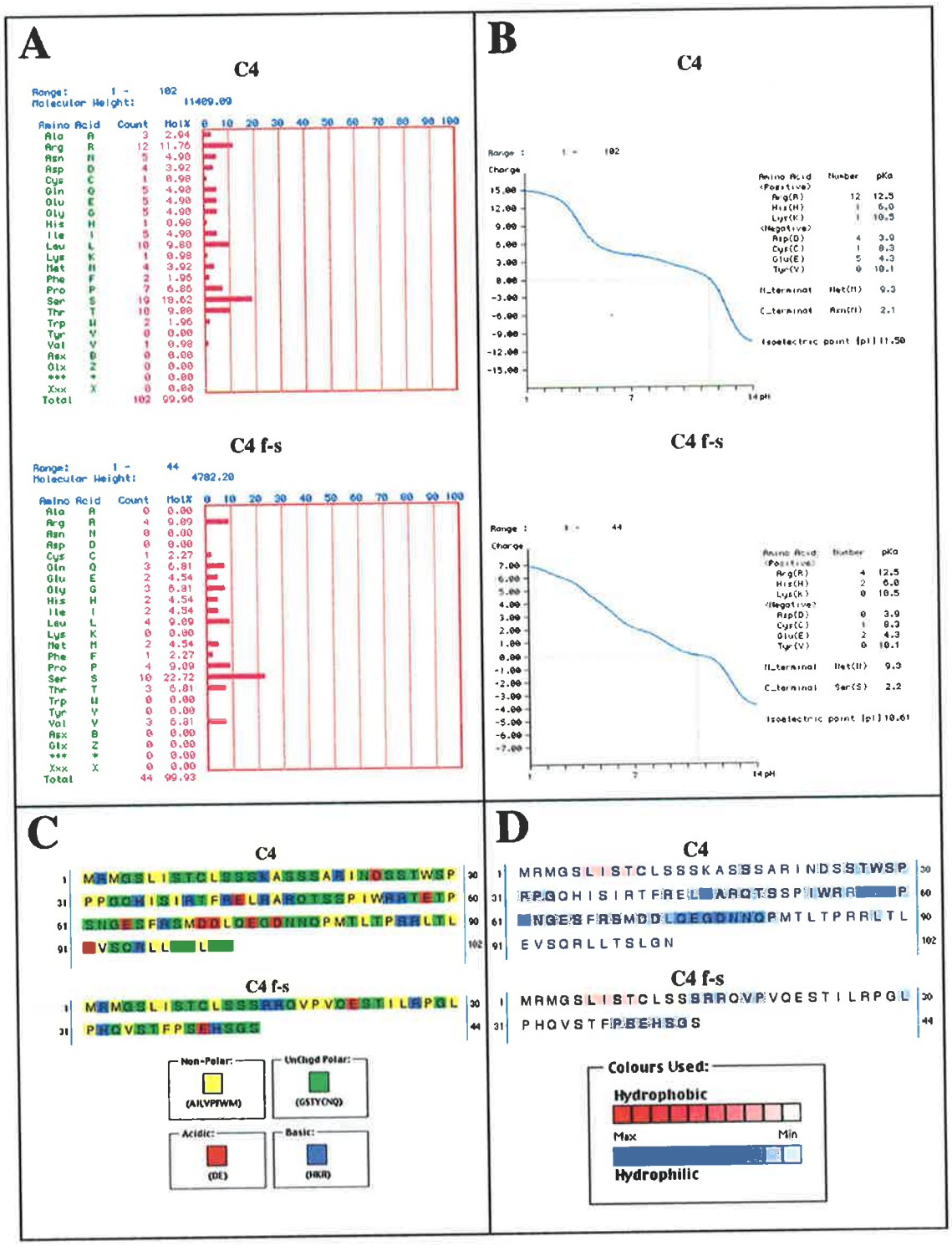


Figure 45. A comparison of the C4 and hypothetical C4 f-s amino acid sequences. The analysis of the amino acid content of each sequence (A), the predicted isoelectric points (B), chemical properities (C) and theoretical hydropathy (D) are shown.

The C4 and hypothetical C4 f-s amino acid sequences were submitted to PSORT (http://psort.nibb.ac.jp) to search for recognisable localisation signals. The amino acid sequences of AC4 from other geminiviruses were submitted for comparison and these findings are summarised in Table 17. The predictions from PSORT indicated the C4 protein from ToLCV may be targeted to the chloroplast stroma (probability of 0.938) or mitochondria (probability of 0.82) whereas the C4 f-s protein may be localised in the mitochondrial intermembrane space (probability of 0.867).

Table 17. Prediction of potential localisation signals (cleavable leader peptide) in the C4 proteins of selected geminiviruses (Tables 2 – 4). The certainty factor is based on 1. The predictions for the C4 of ToLCV and its frame-shift version are shown in bold-type.

Protein	Chloroplast	Mitochondria	Nuclear	Cytoplasm
AbMV AC4	0.975	0.573		
ToLCBV	0.975	0.100		
ChaMV C4	0.969	0.513		
ToLCBDV	0.962	0.519		
SiGMV-Honduras AC4	0.959	0.514		
ToLCV C4	**0.938**	**0.820**		
ICMV C4	0.938	0.676		
CPGMV AC4	0.935	0.444		
ToLCTWV C4	0.895	0.360		
TYLCSV-Almeria C4	0.887	0.676		
TYLCSV-Murcia C4	0.887	0.676		
PYMV-Panama AC4	0.885	0.444		
TYLCV-mild C4	0.884	0.100		
HrCTV C4	0.882	0.100		
TYLCSV-Sicily C4	0.871	0.589		
TPCTV C4	0.854			0.450
ToLCNdV-mild AC4	0.845	0.796		
ToLCNdV-severe AC4	0.845	0.796		
BSCTV C4	0.800	0.100		0.450
BCTV C4	0.800	0.100		0.450
TYLCV C4	0.576	0.469		0.450
TYLCV-Cuba C4	0.575	0.431		0.450
Hypothetical ToLCV C4 f-s	**0.568**	**0.867**		
TYLCSV C4	0.400	0.360		0.450

3.3.6 Relationships between the C4 protein and homologues in other viruses

A comparison of the overall amino acid sequence of C4 from ToLCV with other begomoviruses did not reveal a close relationship with any of viruses shown in Table 18). The similarity between ToLCV-C4 and other monopartite begomoviruses ranged between 58-83% and 61-79% with bipartite begomoviruses. In addition, the alignment of C4 and AC4 sequences shown in Figures 46-48 revealed common blocks of amino acid sequence between unrelated begomoviruses, curtoviruses and a topocuvirus.

Table 18. The percent similarity and identity by GAP analysis of the overall C4 sequence of ToLCV compared with C4 and AC4 sequences of other begomoviruses.

C4	% Similarity	% Identity	AC4	% Similarity	% Identity
AYVV	84	77	ICMV	72	66
ToLCTWV (2748)	84	77	OYVV-PK [201]	72	63
ToLCLV	83	76	PYMV-Trin	66	63
TYLCV-mild	71	65	ToLCNdV-m	66	60
TYLCV-Portugal	71	65	TLCrV	66	61
TYLCV-Murcia	69	64	ACMV	65	55
TYLCV-Almeria	69	63	AbMV	64	60
TbLCJV	69	57	ToLCNdV-s	64	59
TYLCSV-Sicily	68	62	ToMHV	64	55
TYLCSV	65	62	SiGMV-Hond	62	56
TPCTV	64	58	ChaMV	62	54
ToLCBV-Ban4	64	54	PYMV-Panama	61	55
ToLCBDV	62	53	SiGMV-Florida	60	55
ToLCTWV (2739)	61	53	ToLCrV	60	55
TYLCV	58	51	TYLCTHV	59	46
BSCTV	48	38	MYMIV	59	45
BMCTV	42	33	SLCCV	34	20
HrCTV	41	28	EACMV-CM	33	15

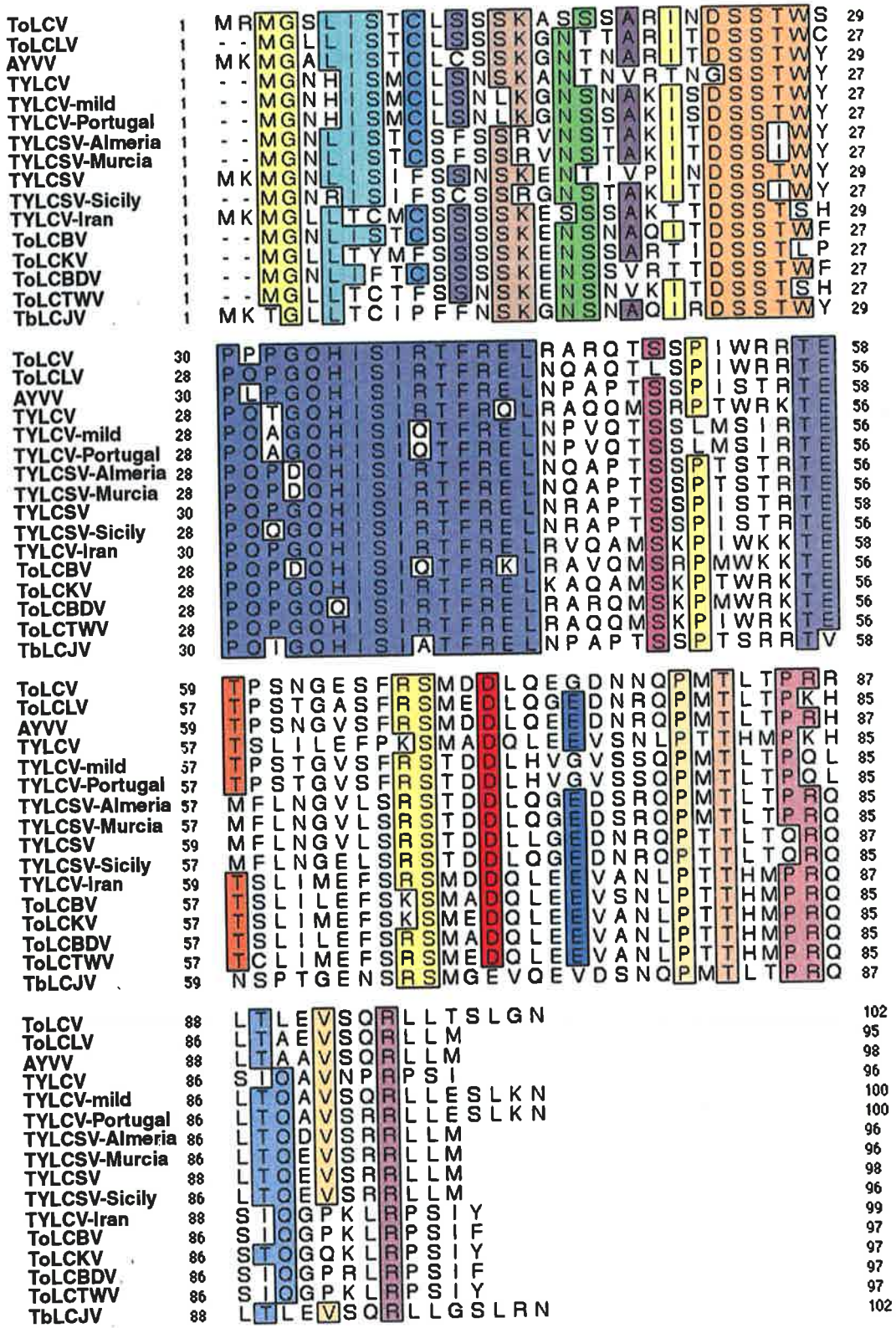


Figure 46. A comparison of the C4 amino acid sequence from different monopartite begomoviruses highlighting common block of identical sequence.

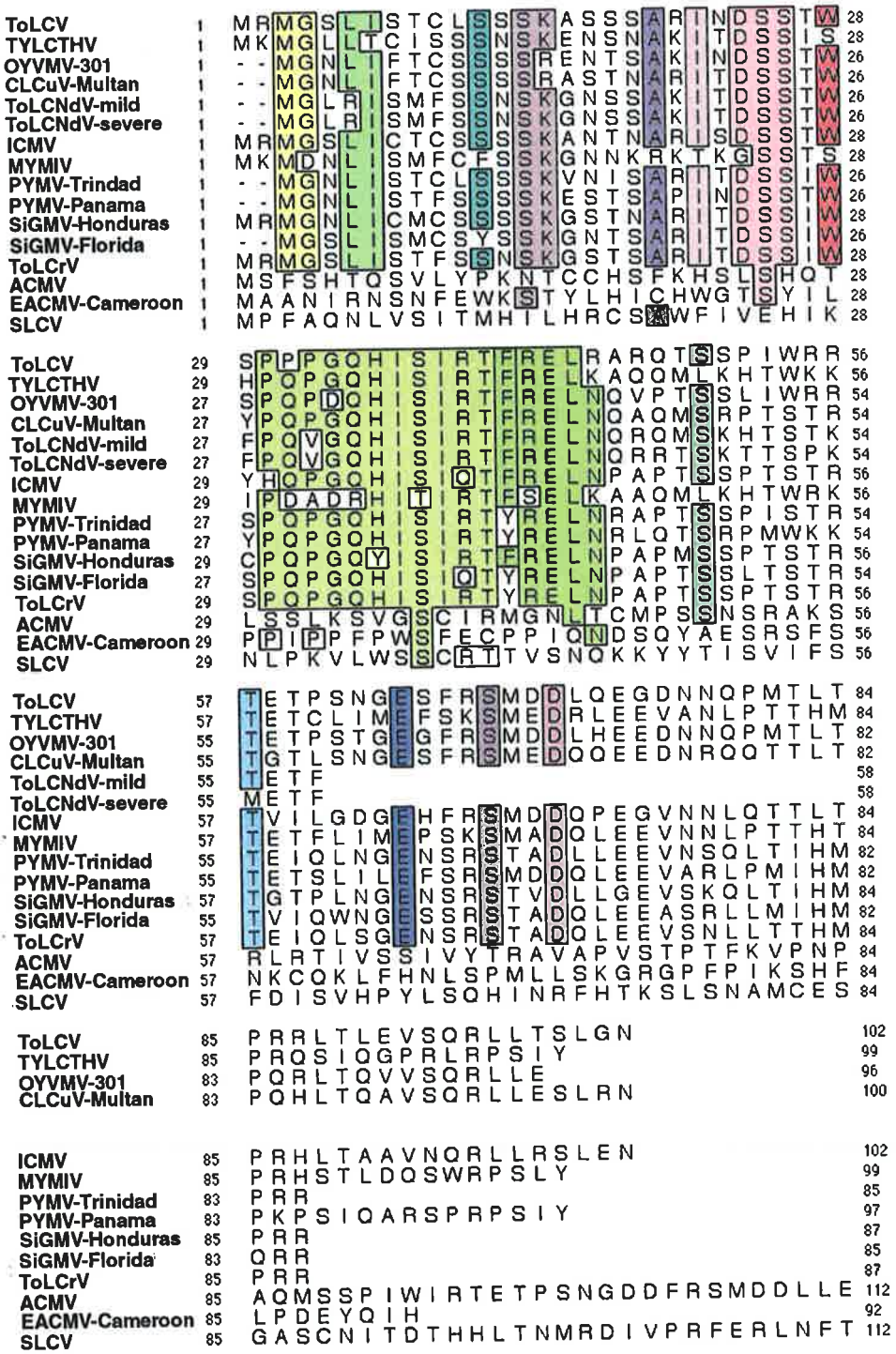


Figure 47. The amino acid sequence of C4 from ToLCV compared with the sequence of AC4 from unrelated bipartite begomoviruses illustrating common blocks of identical amino acid sequence.

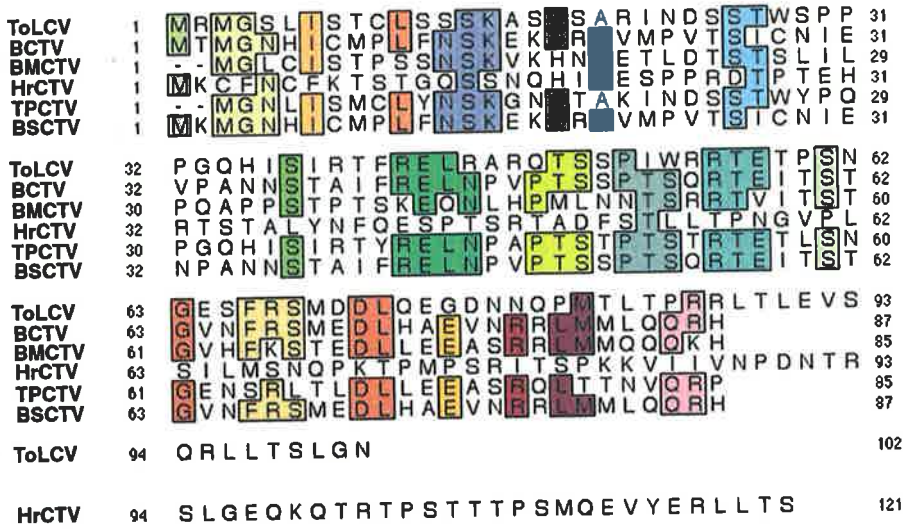


Figure 48. The amino acid sequence of C4 from ToLCV compared with the sequence of C4 from unrelated curtoviruses and a topocuvirus showing common blocks of identical amino acid sequence.

3.3.7 Predicted evolutionary analysis of the *c4* and *ac4* genes of begomoviruses

The predicted evolutionary separation of the *c4* and *ac4* genes of begomoviruses and a topocuvirus (TPCTV) using the phylogeny inference software package (Felsenstein, 1989) is shown in Figure 49. Some regional separation of the genes is evident. The *c4* genes of begomoviruses from Asia separated into two distinct clusters. One cluster included ToLCBDV, ToLCBV, ToLCV-Sri Lanka, TYLCV-Iran, ToLCBV-Ban4, ToLCTWV, and ToLCKV while the other cluster comprised OYVMV-Pakistan 301, ToLCV, AREV, AYVV, TbLCV-Yunnan, ToLCLV, CLCuV-Pakistan, OYVMV-India, and Okra enation virus. Other distinct separations included the American cluster (PYMV, AbMV, ToLCrV, SiGMV) and the viruses from the Mediterranean region (TYLCSV, TYLCSV-Sicily, TYLCSV-Murcia).

The presence of common blocks of amino acid sequence within the C4 and AC4 of unrelated viruses raised the issue whether their existence has been maintained only through the overlapping conserved blocks of the Rep sequences (Figures 50-53). Two of the four biologically important conserved motifs within the Rep of geminiviruses overlap the C4 and AC4 ORFs. These include motif II (HLH_{59}) and motif III ($YXXK_{106}$) that overlap the C4 sequence of ToLCV at $LISTC_{10}$ and $SPIWRR_{56}$, respectively. The block of LIS appears to be generally conserved in the monopartite begomoviruses (Figure 46), whereas LI and I appear to be conserved in bipartite begomoviruses and curtoviruses (Figures 47 and 48, respectively). The C4 and AC4 sequences corresponding to the position of the Rep motif III show a conserved P in the monopartite begomoviruses, no conservation within the corresponding region of bipartite begomoviruses and a PxxxR block within the C4 amino acid sequence of some curtoviruses. Other conserved blocks within the C4 and AC4 sequences appear to be also influenced by the overlapping C1 and AC1 ORFs.

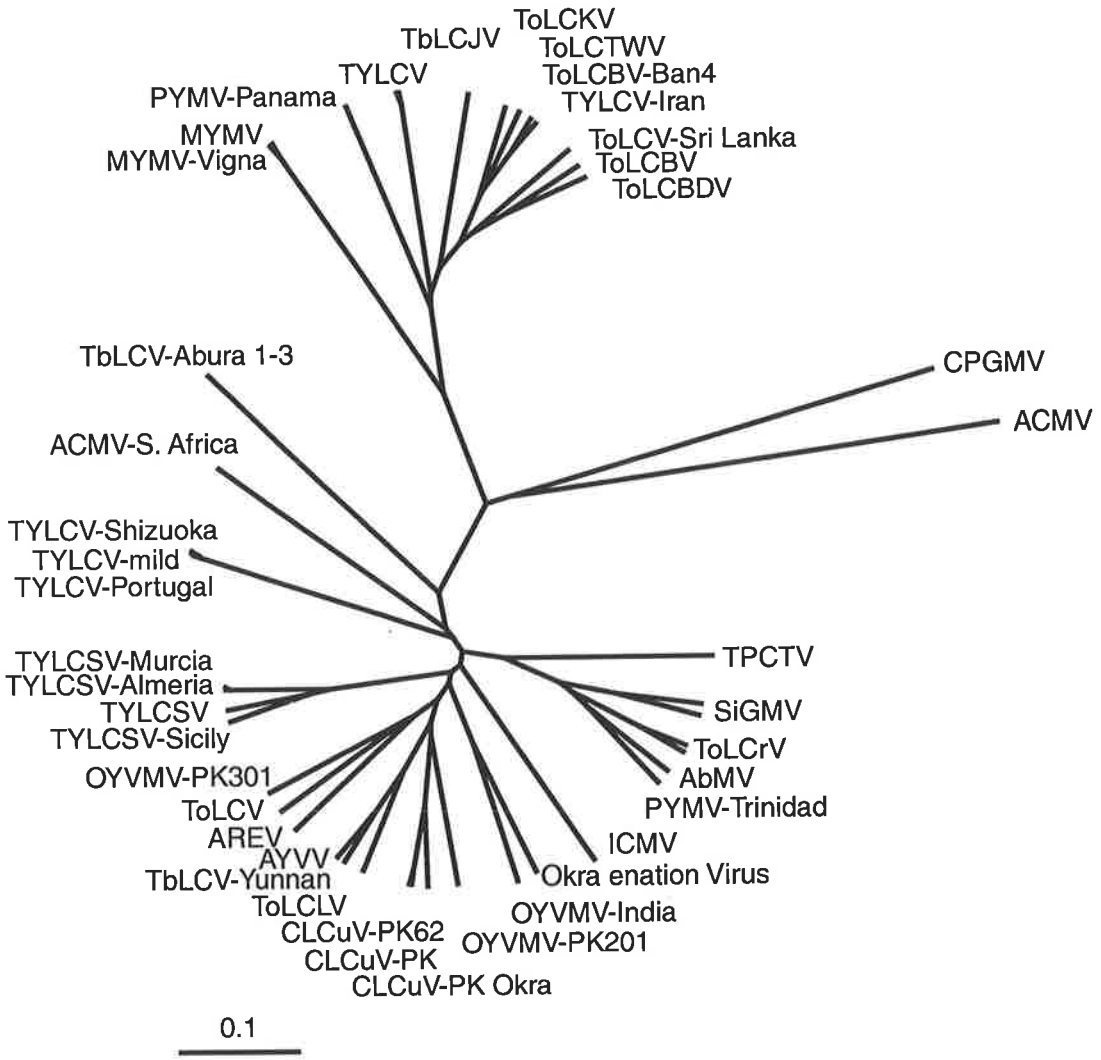


Figure 49. Predicted phylogenic relationships between *c4* and *ac4* genes of geminiviruses derived from a distance matrix processed by the Kitsch protocol of PHYLIP using the Fitch-Margoliash criterion and assuming an evolutionary clock.

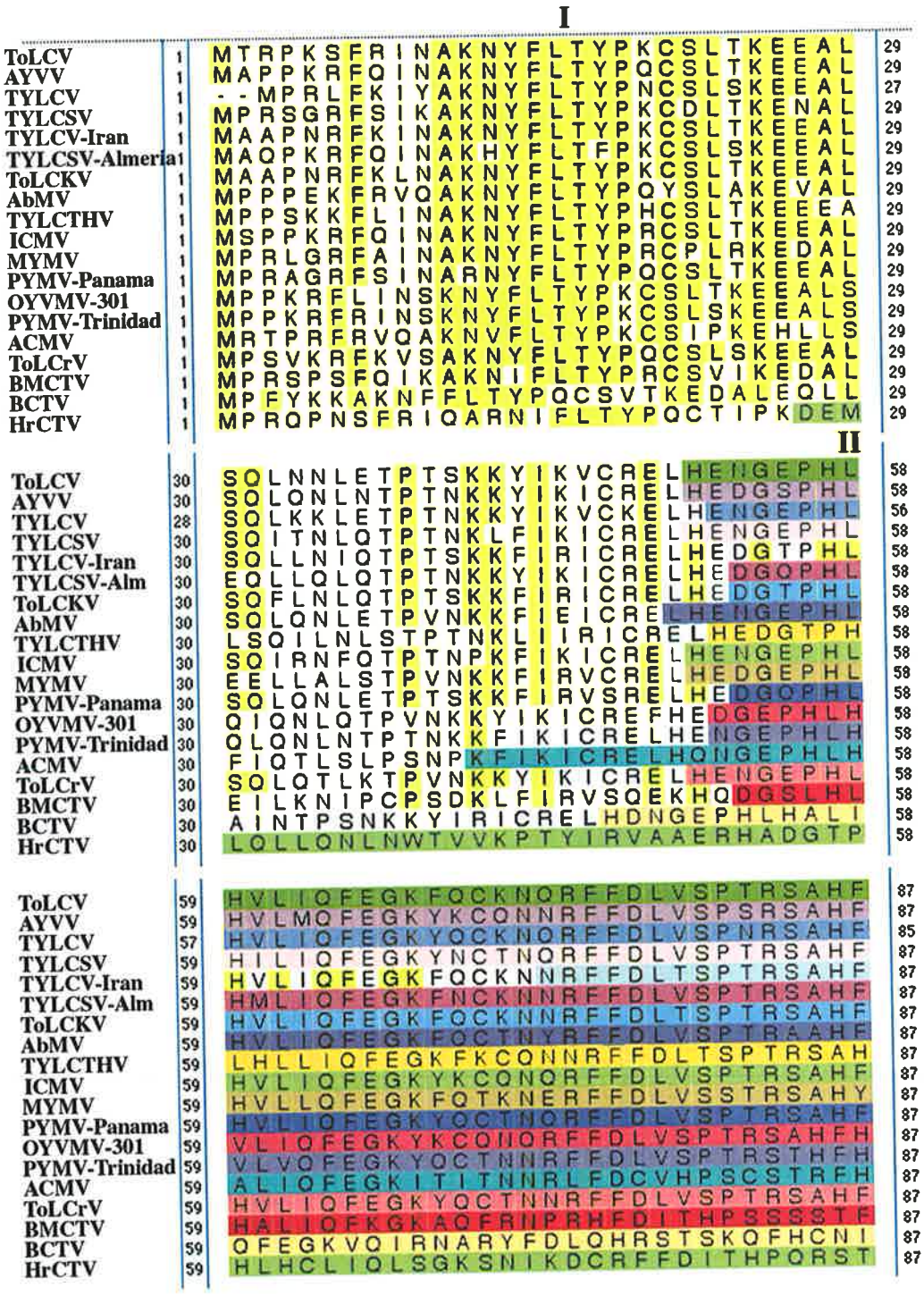


Figure 50. Alignment of the first part of Rep sequences from different monopartite and bipartite geminiviruses. The position of conserved motif I ($FLTY_{18}$) and motif II (HLH_{59}) are shown. The extended coloured bars indicate the location of the respective overlapping C4 or AC4 ORFs relative to common blocks of Rep sequence.

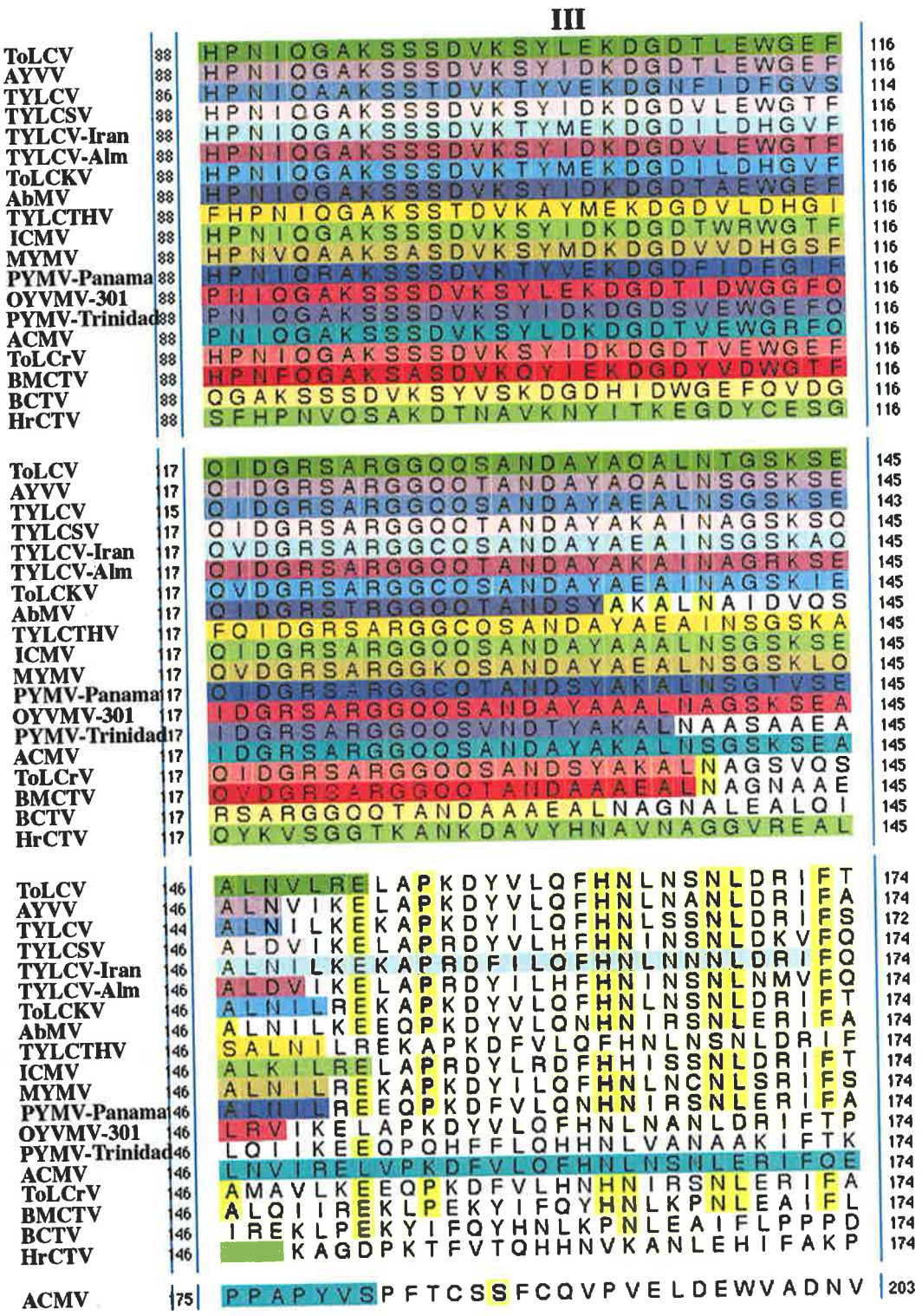


Figure 51. Continuation of the alignment of the Rep sequences from different geminiviruses showing common sequence blocks influencing the overlapping C4 and AC4 ORFs (coloured bars). The position of conserved motif III ($YXXK_{106}$) is shown.

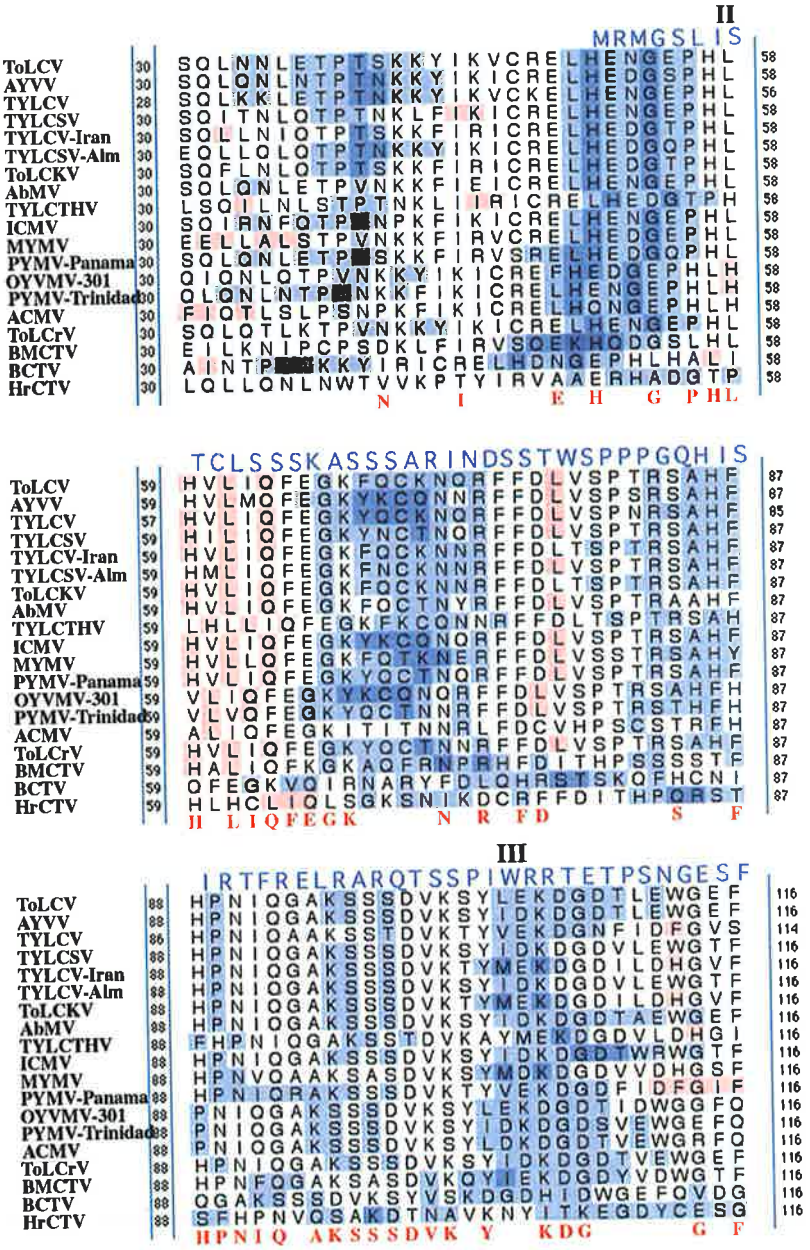


Figure 52A. The theoretical hydropathy of part 1 of the Rep sequence of different geminiviruses that overlap C4 and AC4 sequences. The relative position of motifs II and III are shown with the ToLCV C4 amino acid sequence in blue as a reference. The amino acid sequence shown in red type represents the geminivirus-like sequence found in the genome of *Nicotiana tabacum* (Bejarano *et al.*, 1996).

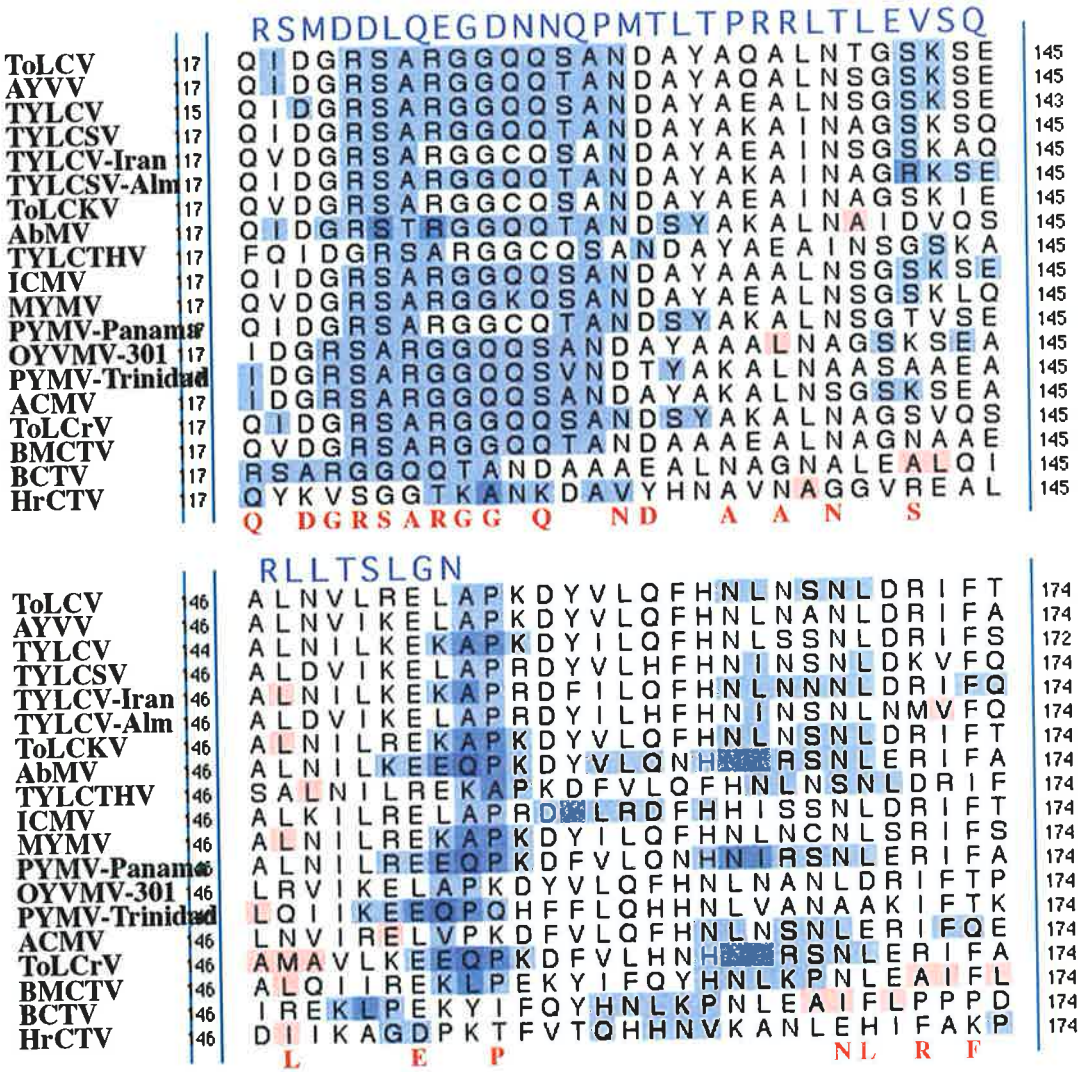


Figure 52B. The theoretical hydropathy of part 2 of the Rep sequence of different geminiviruses that overlap C4 and AC4 sequences. The amino acid sequence shown in red type represents the geminivirus-like sequence found in the genome of *Nicotiana tabacum* (Bejarano *et al.*, 1996).

3.4 Discussion

Genetic modification of *N. tabacum* var. Samsun and *Lycopersicon esculentum* var. Ailsa Craig with insertions of the *c4* gene from an Australian isolate of ToLCV under the control of a 35*S Cauliflower mosaic virus* promoter produced abnormal plant phenotypes resembling virus-infected plants (Figures 29-31). These foliar abnormalities typically included deformed and curled laminae that are similar to those often displayed by virus-infected plants. Some of the transgenic lines also developed tumorous enations on stems, petioles, leaf veins and on the grossly enlarged calyx of deformed flowers (Figures 32-33 and 37-38). These abnormal characteristics were associated with the expression of the *c4* transgene (Figures 39 and 41). In contrast, all the tobacco and tomato plants genetically modified with a frame-shift version of the above gene construction appeared normal in all respects (Figure 34), despite expressing similar levels of transgene RNA as the abnormal 35*S-c4* plants (Figure 40). This demonstrated that the abnormal phenotypes observed were due to the production of the ToLCV C4 protein and not due solely to the presence of the mRNA transcript. The severe abnormalities induced in the 35*S-c4* transgenic plants were most probably due to the over-expression of the C4 protein in different tissues by the powerful 35*S* promoter. In normal plants infected with the wild-type virus, the expression level of the *c4* gene would be controlled by its own promoter (Dry *et al.,* 2000), and by temporal regulation mechanisms.

All of the abnormal characteristics were shown to be heritable and relatively stable (Figure 44). The number of gene inserts integrated into the genome of plants varied from single to multiple insertions as indicated by the ratios of kanamycin resistant plants: kanamycin sensitive seedlings in the T_1 generation (Table 16). Silencing of the

c4 transgene appeared to occur in an occasional shoot on several parental transformed lines that were repeatedly pruned back after each flowering flush and retained for 2 years (Figure 42). Expression of the *c4* transgene was absent in the normalised shoots but detected in remainder of the plant displaying abnormal growth including the young developing leaves.

The results described above support the findings of several studies (Stanley *et al.*, 1992; Stanley and Latham, 1992; Rigden *et al.*, 1994; Latham *et al.*, 1997), using different approaches to study the function of the *c4* gene in different monopartite viruses. In the more recent work (Latham *et al.*, 1997), the expression of 35*S*-*c4* in *N. benthamiana* resulted in abnormal transgenic plant development and the production of tumorigenic enations. The expression of C4 alone played a role in the development of hyperplasia in a permissive host by initiating cell division and tissue proliferation.

Little is known about the function of the C4 protein other than its demonstrated role in cell division. Searches of databases have so far not revealed any significant homologies between C4 of ToLCV and BCTV with known regulatory proteins. Comparison of the overall C4 and AC4 amino acid sequences of different geminiviruses shows the C4 protein of ToLCV to be unique when compared with corresponding homologues in other viruses (Table 18). Closer examination of the alignment of C4 and AC4 amino acid sequences reveals that the C4 of ToLCV does have more conserved blocks of sequence in common with other begomoviruses and the topocuvirus, relative to curtoviruses (Figures 46-48). The predicted evolutionary linkage of *c4* shown in Figure 49, indicates that ToLCV may have closer a relationship with some of the begomoviruses from the South-east Asian region.

As discussed in section **1.5.6**, mutation analyses of AC4 of several bipartite begomoviruses indicated that this ORF was non-functional in the plant hosts studied and thus indicates a fundamental difference with C4 of BCTV and ToLCV. It should be noted that several of the monopartite begomoviruses that cause curling of leaves also develop vein swelling on the abaxial surface of systemically infected leaves (Latham *et al.*, 1997). The proliferation of monopartite begomoviruses may rely on cell division rather than viral movement between cells and C4-mediated cell division may compensate for a lack of DNA-B genes that function in nuclear transport and cell-to-cell movement of bipartite begomoviruses. The C4 protein may induce neighbouring uninfected cells to divide by causing imbalance of phytohormone levels. In support of this, many of the unrelated C4 amino acid sequences appear to have recognisable signals for localisation and transfer to chloroplasts, as predicted by PSORT (Table 15). A certainty factor of 0.938 and 0.8 for chloroplast localisation was obtained for the C4 of ToLCV and BCTV, respectively. In contrast, the chloroplast certainty factor for C4 f-s of ToLCV and TYLCSV were 0.568 and 0.4, respectively. The possible targeting of the C4 protein to either the chloroplast or mitochondria could be resolved through direct comparison of cellular localisation of *c4-gfp* and *c4 f-s-gfp* gene fusions.

It is possible that some of the C4 proteins may also be capable of facilitating intercellular viral movement as has been demonstrated by several viral-encoded proteins involved in viral transport, and as suggested for TYLCSV (Jupin *et al.,* 1994). The expression of the movement protein BC1 from SLCV and TMoV also independently induced virus-like symptoms when expressed in *N. benthamiana* (Pascal *et al.*, 1993; Duan *et al.*, 1997). Direct comparison of amino acid sequences reveals that

C4 of TYLCSV is unrelated to BC1 sequences (data not shown) but TYLCSV shares substantial conserved blocks of sequence with ToLCV (Figure 45).

The overlapping nature of Rep and C4 of ToLCV indicate that they are expressed in a highly regulated manner from a single transcriptional unit (Mullineaux *et al.*, 1993). This arrangement would be essential for coordinating their proposed roles in initiating cellular activities associated with S-phase (Rep) and mitosis (C4). Whether C4 disturbs the balance of phytohormones or has a more direct effect on cyclins or cyclin-dependent kinases or other cell signalling mechanisms needs further clarification by biochemical analysis and further understanding of the viral and host factors with which it interacts. Other viral proteins including the VI of *Cauliflower mosaic virus* (Baughman *et al.*, 1988), p19 and p22 proteins of *Tomato bushy stunt virus* (Scholthof *et al.*, 1995), and ORF 0 protein of *Potato leafroll virus* (van der Wilk *et al.*, 1997), have also induced virus-like symptoms when expressed in transgenic plants. Whether any common mechanism operates between the different viral systems remains to be determined.

Chapter 4

Challenge-inoculation of transgenic plants with ToLCV-DNA

4.1 Introduction

Almost fifteen years have elapsed since the first demonstration of induced viral resistance of transgenic plants expressing the coat protein gene of *Tobacco mosaic virus* (TMV) (Powell-Abel *et al.*, 1986). The inoculated transgenic plants exhibited delayed development of induced viral disease compared with normal plants infected with the wild-type virus. The subsequent application of this discovery has stimulated further studies of pathogen-mediated resistance in a wider range of viruses and has led to a new era of genetic manipulation in plant pathology (Beachy, 1997). Various proposed molecular mechanisms are widely under test for induced pathogen resistance, as suitable genes for the control of economically important pathogens are usually not available for use in conventional breeding programs.

Several classes of viral genes including dysfunctional replicase, movement proteins, non-translatable genes and various antisense gene constructions have been reported to induce varying degrees of synthetic resistance within the host plants (Baulcombe, 1996; Beachy, 1997). In addition, the use of satellite RNAs (Gerlach *et al.*, 1987; Harrison *et al.*, 1987), and defective interfering components (Stanley *et al.*, 1990) have been studied and recommended in some cases. The expression of virus-derived sense and/or antisense RNA in transgenic plants has been used to confer RNA-mediated resistance against specific viruses (Dougherty *et al.*, 1994; Smith *et al.*, 1994; Baulcombe, 1996; Goodwin *et al.*, 1996). More recently, mechanisms based on homology-dependent post-transcriptional gene silencing have been proposed and actively studied in several labs throughout the world (Stam *et al.*, 1997; Waterhouse *et al.*, 1998; Smith *et al.*, 2000).

The ability of *Agrobacterium* to transfer cloned infectious DNA into plants (Grimsley et al., 1986) has proved to be a major technical advance in the study of non-mechanically transmissible geminiviruses. Infectious constructs can be made with as little as 1.1 copies of the viral genome. Gene constructions containing two intergenic regions infect plants with a higher efficiency than those constructs with only one (Elmer *et al.,* 1988b) and this method has been used extensively to test transgenic plants for resistance to viral DNA by challenge-inoculation.

Although some resistance to specific geminiviruses has been reported in transgenic plants using several different strategies (**1.9.2**) including the expression of sense or antisense *ac1* or *c1* sequence in plants, no attempts to test transgenic plants expressing the *c4* gene have been reported. The aim of the following work was to test all of the transgenic plants regenerated during this course of study by challenge-agroinoculation with ToLCV-DNA, to assess the potential resistance of these plants to the homologous virus.

4.2 Experimental procedure

4.2.1 Preparation of the inoculum

An overnight culture of *A. tumefaciens* strain LBA4404 (**2.5.1.2**) containing a recombinant pBin19 plasmid harbouring a 1.5 mer of the ToLCV genome or a 1.5 mer of a *c4* defective mutant of ToLCV (Rigden *et al.,* 1994) was made for each batch of plants to be tested. Each bacterial culture used as an inoculum was adjusted to an optical density of 0.56 units at 600 nm. Each culture was initially diluted to determine the minimum dilution of bacterial cells required for a near 100% infection of agro-inoculated tobacco and tomato plants using aliquots diluted 10-fold serially to give a range of cultures from 10^{-1} to 10^{-6}. A further aliquot of 10 µl of each dilution was spread onto culture plates containing kanamycin and rifampicin (**2.5.1.2**). The number of colonies that developed after incubation at 28°C for 48 hours was recorded.

Young plants of native *N. tabacum* var. Samsun and *Lycopersicon esculentum* var. Ailsa Craig were separately agro-inoculated at the six to eight leaf stage with 10 µl of one of the serially diluted bacterial cultures prepared as above. The inoculation was done with a micro-syringe by injecting 1 µl into 10 positions in the leaf axils of each plant. The inoculated plants were maintained in a glasshouse on trays to collect any bacterial contaminated effluent and observed daily for symptoms of disease as shown in Figures 29 and 17.

4.2.2 Dot-blot hybridisation

A sample of 50 mg tissue from young leaves of the virus-inoculated plants were extracted with alkali (**2.5.11.3**) and a 5 µl aliquot of each DNA preparation was separately dot-blotted into a 1 cm square of a grid marked on Zeta-Probe membrane.

The membrane was processed (**2.5.11.3**, **2.5.11.6** and **2.5.11.7**) using a linearised full-length fragment of ToLCV-DNA as a template for the synthesis of the ^{32}P-labelled probe. When required, the probed nylon membrane was cut into individual 1 cm squares that contained a separate spot of the hybridised DNA sample. Each individual square of membrane was transferred to a vial containing 10 ml of liquid Ready-Solv EP scintillation cocktail and the radioactivity counted for 10 minutes in a Beckman LS 3801 Liquid Scintillation Counter.

4.2.3 Evaluation of virus induced symptoms

The inoculated plants were inspected daily in most cases and their foliar symptoms were assessed using an arbitrary rating system based on:

0 = symptomless plants

0.5 = initial signs of disease (1 pair of newly developing leaves)

1 = mild disease

2 = moderate disease

3 = severe disease

Statistical analyses were done with the assistance of CSIRO Mathematical & Information Sciences, Adelaide, South Australia to determine significant differences between the selected transformed lines and the untransformed control plants. The treatments were compared and evaluated on the following basis:

(a) numbers of plants that developed virus symptoms out of the total inoculated plants;

(b) the calculated arithmetic mean infection, standard deviation, and analysis of variance obtained from the summed scores of symptoms per treatment;

(c) a logistic regression analysis on the binomial data using a similar technique as shown in Modern Applied Statistics with S-Plus by W. N. Venables and B. D. Ripley (3rd Edition Springer-Verlag New York) under 7.2 Binomial data on page 218.

4.3 Results

4.3.1 Determination of threshold levels for agro-infection

The ability of *Agrobacterium* to transfer infectious DNA into plants (Grimsley *et al.*, 1986) was used to challenge-inoculate the transformed plants with copies of the ToLCV genome to determine their relative sensitivity to viral infection. The findings shown in Table 19 indicated that an inoculum concentration equivalent to or higher than the 10^{-4} dilution of the standardised culture (**4.2.1**) was needed to achieve a 100% infection rate of the inoculated plants. A 10 µl aliquot of the 10^{-4} dilution of the standardised inoculum was found to produce ~2,200 bacterial colonies when spread on plates containing kanamycin and rifampicin (**2.5.1.2**).

Table 19. The relative infection rates of tobacco (cv. Samsun) and tomato (cv. Ailsa Craig), following agro-infection with dilutions of the standardised inoculum.

Host	Inoculum (10 µl)	Numbers of plants symptomatic/total plants inoculated		
		16 days	23 days	32 days
Samsun	10^{-5}	0/10	4/10	7/10
Samsun	10^{-4}	0/10	2/10	10/10
Samsun	10^{-3}	8/10	10/10	10/10
Ailsa Craig	10^{-5}	0/12	5/12	7/12
Ailsa Craig	10^{-4}	11/12	11/12	12/12
Ailsa Craig	10^{-3}	12/12	12/12	12/12

The standardised inoculum diluted to 10^{-3} induced earlier development of symptoms and more dramatic stunting of plants, whereas a dilution of 10^{-5} did not achieve high levels of infection. In the studies that follow, a standardised inoculum diluted to 10^{-4} was used routinely and where indicated, a higher concentration of 10^{-3} was tested.

4.3.2 Agro-inoculation of 35*S-c4* transformed tobacco

4.3.2.1 Comparison of ToLCV-DNA and C4 ORF mutant DNA inocula

Selected T_1 generation seedlings (35*S-c4*) from two transgenic tobacco lines (S5 and Alpha-1) that displayed an apparent normal phenotype were compared with three transgenic lines with a moderately abnormal phenotype and wild-type Samsun control plants in Test 1. These plants were agro-inoculation with either ToLCV-DNA or a C4 ORF mutant version of ToLCV-DNA (**4.2.1**). In previous experiments (Rigden *et al.*, 1994), a high concentration of the C4 ORF mutant ToLCV-DNA inoculum had induced milder symptoms in wild-type Samsun plants compared with those inoculated with ToLCV-DNA. In this test, each of the plants were inoculated with a standardised inoculum diluted to 10^{-4} concentration (**4.2.1**), and evaluated using the 0-3 rating system described in **4.2.3**. The purpose of this test was to test selected transgenic lines for resistance against ToLCV-DNA infection and for their ability to complement the mutant C4 ORF in the inoculum, as indicated by the response of a wild-type level of disease in the inoculated plants.

Following agro-inoculation, the symptoms displayed by plants were evaluated at 19, 27, 40 and 47 days, post-inoculation (p-i). Evaluation of the symptoms induced in Samsun tobacco by ToLCV revealed initial symptoms at 19 days, followed by a general increase in the severity of symptoms over time (0.5-2 rating), with maximum development occurring between 27 and 40 days p-i (Table 20). By contrast, the Samsun plants inoculated with the C4 ORF mutant virus started to develop the first signs of disease (0.5 rating) at 19 days p-i, followed by apparent recovery. The P3, S5, Alpha-1 transgenic lines and untransformed Samsun plants also began to show viral symptoms at 19 days p-i.

Table 20. Comparison of symptoms induced in Samsun tobacco plants and selected T_1 transgenic 35*S-c4* lines following agro-inoculation (10^{-4} dilution) with ToLCV-DNA or a C4 ORF mutant version of ToLCV (**Test 1**). The numbers of plants symptomatic (0-3 rating - section **4.2.3**) out of the total plants inoculated are indicated in parenthesis.

Variety	ToLCV-DNA inoculum				C4 ORF mutant DNA inoculum			
	19 days	27 days	40 days	47 days	19 days	27 days	40 days	47 days
Samsun	0.5, 0.5 1, 0.5, 0.5, 1, 0.5, 1, 0.5, 0.5 0.5, 0.5 0.5, 0.5 1, 0.5, 0.5 0.5, 0.5, (19/19)	0.5, 0.5 0.5, 0.5 2, 0.5, 0.5, 1, 0.5 (9/9)	2, 2, 2, 2, 2, 2, 2, 2, 2, 2 (9/9)	2, 2, 2, 2, 2, 2, 2, 2, 2, 2 (9/9)	0.5, 0.5 0.5, 0.5 0.5, 0.5 0, 0.5, 0.5, 0.5 (9/10)	0, 0, 0, 0, 0, 0, 0, 0, 0, 0 (0/10)	0, 0, 0, 0, 0, 0, 0, 0, 0, 0 (0/10)	0, 0, 0, 0, 0, 0, 0, 0, 0, 0 (0/10)
S5	1, 1, 1, 1, 0.5, 0.5, 0.5, 0.5 0.5, 1, 0.5 (11/11)	2, 2, 1, 1, 0.5, 0.5, 0.5 1, 0.5, 0.5, 0.5 (11/11)	2, 2, 2, 2, 2, 2, 2, 2, 2, 2 (11/11)	2, 2, 2, 2, 2, 2, 2, 2, 2, 2 (11/11)	0, 0, 0, 0, 0, 0, 0, 0, 0, 0 (0/10)	0, 0, 0, 0, 0, 0, 0, 0, 0, 0 (0/10)	0, 0, 0, 0, 0, 0, 0, 0, 0, 0 (0/10)	0, 0, 0, 0, 0, 0, 0, 0, 0, 0 (0/10)
Alpha1	1, 0.5, 0.5, 0.5 1, 1, 0.5, 0.5 1, 1 (10/10)	1, 0.5, 0, 0.5, 1, 2, 0, 0.5, 1, 2 (8/10)	2, 2, 1, 2, 2, 2, 1, 2, 2, 2 (10/10)	2, 2, 1, 2, 2, 2, 1, 2, 2, 2 (10/10)	0, 0, 0, 0, 0, 0, 0, 0, 0, 0 (0/10)	0, 0, 0, 0, 0, 0, 0, 0, 0, 0 (0/10)	0, 0, 0, 0, 0, 0, 0, 0, 0, 0 (0/10)	0, 0.5, 0.5, 0.5 0.5, 0, 0.5, 0, 0, 0, 0, (5/10)
P3	1, 1, 1, 1, 1, 1, 1, 1 (8/8)	2, 1, 1, 1, 0.5, 1, 1, 1 (8/8)	2, 2, 2, 2, 2, 2, 2, 1 (8/8)	2, 2, 2, 2, 2, 2, 2, 1 (8/8)	0, 0.5, 0.5, 0.5 0, 0, 0.5, 0.5 0.5, 0.5 0.5 0.5, 0.5 0.5 (12/15)	0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0.5 (1/15)	1, 1, 1, 1, 1, 1, 0.5, 1, 0.5, 1, 0.5, 0.5 0.5, 0.5 0.5 (15/15)	1, 1, 1, 1, 1, 1, 0.5, 1, 0.5, 0.5 0, 0.5, 0, 0, 0 (11/15)
X5	0, 0, 0, 0, 0, 0 (0/6)	0, 0.5, 0, 0, 0, 0 (1/6)	1, 1, 0.5, 0.5 1, 0 (5/6)	1, 1, 0.5, 0.5 1, 0 (5/6)	0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0 (0/15)	0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0 (0/15)	0.5, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0 (1/15)	0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0 (0/15)
B3	0.5, 0, 0.5, 0, 0, 0, 0, 0 (2/7)	0.5, 0, 0, 0, 0, 0, 0, 0 (1/7)	1, 1, 1, 1, 1, 1, 1 (7/7)	1, 1, 1, 1, 1, 1, 1 (7/7)	0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0 (0/16)	0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0 (0/16)	0, 0.5, 0.5, 0.5 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0 (3/16)	0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0 (0/16)

In comparison, the same symptom severity did not develop in the X5 and B3 transgenic lines until 27 and 40 days p-i, indicating that these plants had some ability to resist infection by ToLCV-DNA. The statistical analysis of the Samsun and selected T_1 progeny of 35*S-c4* transgenic tobacco plants (Tables 21) indicated a possible variety by day interaction ($P = 0.046$) and confirmed a highly significant variety and day effect ($P< 0.000$). The significant variety effect is due to the consistently higher level of symptom displayed by the varieties P3, S5, Alpha1 and Samsun and the consistently lower level of symptom displayed by B3 and X5 over the tested period. The overall means of varieties are ranked as {P3 ≥ S5 ≥ Alpha1 ≥ Samsun} ≥ B3 ≥ X5.

Table 21. Analysis of variance (data in Table 20) on symptoms displayed by plants agro-inoculated with ToLCV-DNA at 0.56 x 10^{-4} OD_{600} dilution (**Test 1**).

Source	DF	Sequential SS	Adjusted SS	Adjusted MS	F	Probability (P)
Varieties	5	31.7111	33.3195	6.6639	61.16	0.000
Days	3	56.6861	48.2448	16.0816	147.6	0.000
Varieties x Days	15	2.8467	2.8467	0.1898	1.74	0.046
Error	190	20.7011	20.7011	0.1090		
Total	213	111.9451				

The plants inoculated with the defective C4 ORF mutant of ToLCV-DNA (Rigden *et al.,* 1994) showed either no symptoms of infection or lower percentages of infected plants than corresponding plants infected with wild-type virus (Table 20). The P3 transgenic line inoculated with the C4 ORF mutant inoculum behaved somewhat like untransformed Samsun by displaying the first signs of disease development at 19 days p-i (80 and 90% infection, respectively), followed by apparent recovery. The C4 ORF mutant-inoculated P3 line differed from similarly inoculated Samsun by displaying ToLCV disease 40 days p-i. A recovery of symptoms in P3 (73% symptomatic) was

again apparent at 47 days p-i. Similarly, 6% and 18% of selected X5 and B3 showed initial symptoms and recovery during the period of 40 and 47 days p-i. Also, 55% of the C4 ORF mutant inoculated Alpha-1 plants displayed the initial symptoms of disease at 47 days p-i. The low incidence of symptoms induced by the C4 ORF mutant viral DNA indicated that a higher concentration of the inoculum should be used to evaluate the effects of mutant virus infection on the transgenic plants.

In Test 2, the same plant treatments were inoculated with a 10-fold increase in the concentration of the C4 ORF mutant inoculum (10^{-3} dilution) and were evaluated at 21, 40 and 51 days p-i, as shown in Table 22. These inoculated transgenic tobacco lines generally had a lower percentage of plants showing symptoms compared with the untransformed Samsun plants. Some of the P3 and Alpha1 plants appeared to recover from the early signs of disease symptoms. The development of symptoms of the X5 and B3 plants increased progressively with time although the onset of disease was significantly delayed compared with the inoculated Samsun plants. It is possible that complementation of the C4 ORF mutation of the replicating viral DNA may have occurred in some of the transgenic plants expressing the *c4* gene. Reversion of mutations back to the wild-type state, occurs frequently in plants that express wild-type symptoms, as discussed in section **1.5.6**.

Table 22. Comparison of symptoms (0-3) induced by the C4 ORF mutant viral DNA (10^{-3} dilution) in untransformed Samsun tobacco and selected T_1 transgenic C4 lines (**Test 2**). The numbers of plants infected out of the total plants inoculated are indicated in parenenthesis.

Variety	Rating of symptoms induced by inoculated plants		
	21 days	40 days	51 days
Samsun	1, 0, 1, 1, 1, 0.5, 1, 0, 0, 0, (6/10)	2, 0.5, 1, 1, 1, 0, 0.5, 0, 0.5, 0, (7/10)	2, 1, 2, 2, 1, 0.5, 2, 0.5, 0.5, 0, (9/10)
S5	0, 0.5, 0, 0, 0, 0, 0.5, 0.5, 0, 0, (3/10)	0, 2, 0, 0, 0, 0, 0.5, 0.5, 0, 0, (3/10)	0, 2, 0, 0, 0, 0, 0.5, 2, 0, 0, (3/10)
Alpha 1	0, 1, 0, 0, 0, 0.5, 1, 0, 0.5, 0.5, (5/10)	0, 0.5, 0, 0, 0, 0, 0.5, 0, 0, 0.5, (3/10)	0, 1, 0, 0, 1, 0.5, 2, 0, 0, 1, (5/10)
P3	1, 0.5, 0.5, 0.5, 1, 1, 0.5, 0.5, 0.5, 1, 0.5, 0.5, 0.5, 0.5, 1, (15/15)	1, 0, 0, 0.5, 0.5, 1, 0, 0, 0, 0, 0, 0, 0, 0, 0, (4/15)	2, 1, 0, 2, 2, 2, 0.5, 0, 0, 0.5, 0.5, 0, 0, 0, 0.5, (9/15)
X5	0, 0, 0, 0, 0, 0, 0, 0, 0.5, 0, 0, 0, 0, 0, 0, 0, (1/15)	0, 0, 0, 0, 0, 0, 0, 0, 0.5, 0.5, 0, 0, 0, 0, 0, (2/15)	1, 0, 0, 0, 0, 0, 0, 0, 1, 0.5, 0, 0, 0.5, 0, 0, (4/15)
B3	0.5, 0, 0.5, 0.5, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, (3/16)	0.5, 0, 0.5, 0.5, 0.5, 0, 0.5, 0, 0, 0, 0, 0, 0, 0.5, 0, 0, (6/16)	1, 0, 1, 1, 1, 0, 1, 0, 0.5, 0, 0, 0, 0, 1, 0, 0, (7/16)

Statistical analysis of the symptoms induced by the C4 ORF mutant inoculum on day 21 p-i (Table 23) reveals a highly significant variety effect ($P < 0.001$) due to P3 ≥ Samsun ≥ Alpha-1 ≥ {S5, B3, X5}. The analysis of symptoms on day 40 p-i also reveals a significant variety effect ($P = 0.017$) due to Samsun ≥ {S5, P3, B3, Alpha-1} ≥ X5. Likewise, statistical analysis of symptoms on day 51 also shows a significant variety effect ($P = 0.022$) due to Samsun ≥ P3 {Alpha-1, S5, B3} ≥ X5.

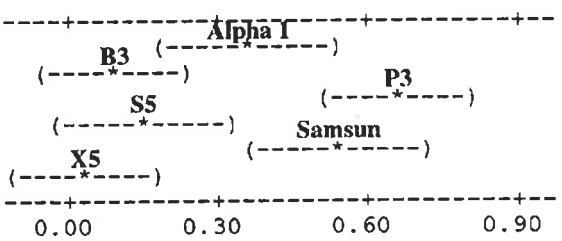
Table 23. Analysis of variance on the rating of disease symptoms for the transformed tobacco lines and untransformed Samsun tobacco inoculated with the C4 ORF mutant-DNA inoculum (10^{-3} dilution). Disease symptoms were evaluated at 21, 40 and 51 days post-inoculation. The lower panel shows the Mean, Standard Deviation (95% confidence interval) derived from summed scores of symptoms per treatment.

Test 2 - 21 days post-inoculation

Source	DF	SS	MS	F	*Probability*
Variety	5	4.6378	0.9276	10.91	0.000
Error	70	5.9510	0.0850		
Total	75	10.5888			

Level	N	Mean	StDev
Alpha 1	10	0.3500	0.4116
B3	16	0.0937	0.2016
P3	15	0.6667	0.2440
S5	10	0.1500	0.2415
Samsun	10	0.5500	0.4972
X5	15	0.0333	0.1291

Pooled StDev = 0.2916

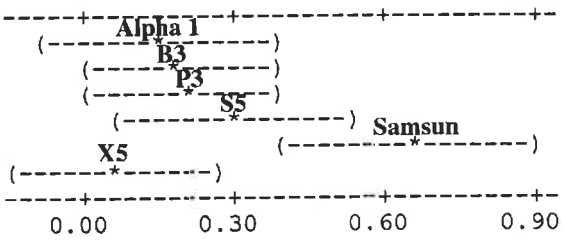


Test 2 - 40 days post-inoculation

Source	DF	SS	MS	F	*Probability*
Variety	5	2.316	0.463	2.97	0.017
Error	70	10.921	0.156		
Total	75	13.237			

Level	N	Mean	StDev
Alpha 1	10	0.1500	0.2415
B3	16	0.1875	0.2500
P3	15	0.2000	0.3684
S5	10	0.3000	0.6325
Samsun	10	0.6500	0.6258
X5	15	0.0667	0.1759

Pooled StDev = 0.3950

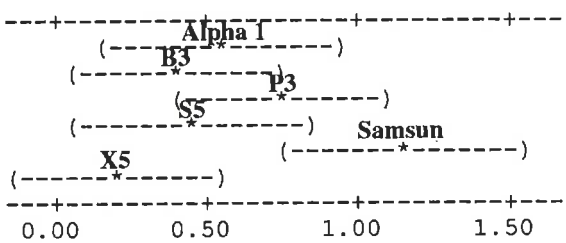


Test 2 - 51 days post-inoculation

Source	DF	SS	MS	F	*Probability*
Variety	5	6.372	1.274	2.84	0.022
Error	70	31.418	0.449		
Total	75	37.789			

Level	N	Mean	StDev
Alpha 1	10	0.5500	0.6852
B3	16	0.4062	0.4905
P3	15	0.7333	0.8423
S5	10	0.4500	0.8317
Samsun	10	1.1500	0.7835
X5	15	0.2000	0.3684

Pooled StDev = 0.6699



4.3.2.2 Challenge-inoculation of 35*S-c4* tobacco with ToLCV-DNA

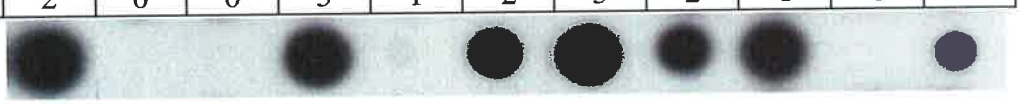
The following experiments (Tests 3-8) were carried out to extend the range of lines tested and to determine the repeatability of potential resistance to ToLCV infection exhibited by some T_1 progeny of the 35*S-c4* transgenic lines. The viral inoculum was diluted to either 10^{-4} or 10^{-3} concentration and in all cases the inoculated plants were first evaluated at 21 days following inoculation.

In Test 3, an inoculum diluted to 10^{-4} concentration was used to agro-infect Samsun control plants, selected 35*S-c4* T_1 transgenic lines (B1, B3, D1, D2, G1, P2, Q3, R1, X5) and a line transformed with the pBin19 vector alone (Bin). Evaluation of plants at 21 and 28 days post-inoculation showed that 100% of the Samsun and plants transformed with the control Bin19 vector developed disease symptoms. By comparison, only 59% and 67% of the total 35*S-c4* transgenic plants developed symptoms at day 21 and 28, respectively. Detection of viral DNA in the inoculated transformed tobacco plants by dot-blot hybridisation under uniform conditions and exposure is also shown in Figure 53. The ToLCV-DNA was detected in 100% of the inoculated Samsun plants and plants of the Bin B5 line at 28 days p-i, whereas the viral DNA could only be detected in 74% of the individual inoculated 35*S-c4* transgenic plants. This indicated that some of the individual plants of the 35*S-c4* transgenic lines appeared to be more tolerant of the challenge-inoculation than others, as indicated by no or lower rates of DNA replication and ameliorated disease symptoms. Statistically, the differences in the severity of symptoms expressed between the groups were significant at the 5% level at 21 days post agro-inoculation (Table 24), while at 28 days post-inoculation the differences between lines were significant at the 1% level.

Figure 53. Comparison of the severity of symptoms and levels of viral DNA by dot-blot hybridisation expressed by untransformed Samsun (Sam) and T_1 progeny of transformed 35*S-c4* tobacco lines following infection a 10^{-4} diluted ToLCV-DNA/*Agrobacterium* culture (**Test 3**). Symptoms were evaluated using the 0-3 scale described in section **4.2.3**.

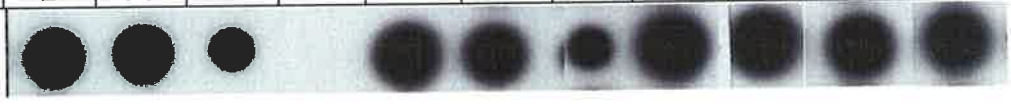
P-i	Sam	Sam	Sam	Sam	Sam	Sam	Bin	Bin	Bin	Bin	Bin
21 days	3	3	2	2	2	2	3	2	2	2	2
28 days	3	3	3	2	3	2	3	2	2	3	2

P-i	P2	P2	P2	D1	D1	D1	R1	R1	R1	R1	R1
21 days	2	0	0	3	0	0.5	3	2	1	0	0
28 days	2	0	0	3	1	2	3	2	1	0	0



P-i	R1	B3	B3	B3	B3	B3	B3	X5	X5	X5	X5
21 days	0	2	0	0	1	0.5	0.5	2	1	0.5	0.5
28 days	0	2	0	2	0.5	0.5	0	1	0.5	0	2

P-i	X5	X5	Q3	Q3	Q3	B1	B1	B1	B1	B1	B1
21 days	2	0	0	2	2	2	2	0	2	2	1
28 days	2	0.5	0	1	2	2	1	0	3	1	1



P-i	G1	G1	G1	G1	D2	D2
21 days	3	0	0	0	0	0
28 days	2	0	1	0	1	0

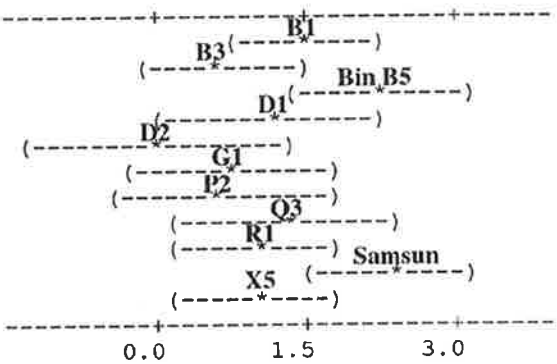
Table 24. Analysis of variance for symptoms displayed by plants at 21 and 28 days post-inoculation with a 10^{-4} diluted ToLCV-DNA/*Agrobacterium* culture. The lower panel shows the Mean, Standard Deviation (95% confidence interval) derived from summed scores of symptoms per treatment as shown in Figure 53.

Test 3 - 21 days post-inoculation

Source	DF	SS	MS	F	*Probability*
Variety	10	19.908	1.991	2.09	0.05
Error	39	37.217	0.954		
Total	49	57.125			

Level	N	Mean	StDev
B1	6	1.5000	0.8367
B3	6	0.6667	0.7528
Bin B5	5	2.2000	0.4472
D1	3	1.1667	1.6073
D2	2	0.0000	0.0000
G1	4	0.7500	1.5000
P2	3	0.6667	1.1547
Q3	3	1.3333	1.1547
R1	6	1.0000	1.2649
Samsun	6	2.3333	0.5164
X5	6	1.0000	0.8367

Pooled StDev = 0.9769

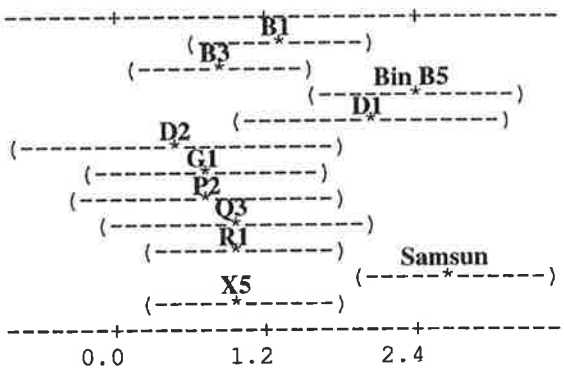


Test 3 - 28 days post-inoculation

Source	DF	SS	MS	F	*Probability*
Variety	10	24.903	2.490	2.89	0.008
Error	39	33.617	0.862		
Total	49	58.520			

Level	N	Mean	StDev
B1	6	1.3333	1.0328
B3	6	0.8333	0.9309
Bin B5	5	2.4000	0.5477
D1	3	2.0000	1.0000
D2	2	0.5000	0.7071
G1	4	0.7500	0.9574
P2	3	0.6667	1.1547
Q3	3	1.0000	1.0000
R1	6	1.0000	1.2649
Samsun	6	2.6667	0.5164
X5	6	1.0000	0.8367

Pooled StDev = 0.9284



In Test 4, the analysis of variance for symptoms induced in the tobacco lines by the lower level of inoculum showed no significant difference (P = 0.065) between the transgenic lines and Samsun tobacco (Table 25). Virus infection rates of 100% were obtained in Samsun, and in transgenic lines including Bin, B1, B2, B3, E1, L3, P1, P3, Q1, S5, W1. In contrast, lower rates of infection occurred in some transgenic lines ranging from 0% (R1) to 33% (G1, P2, Q3) or 66% (D1 and X5). The analysis of symptoms (Table 26) displayed by replicates of the same transgenic lines inoculated with the higher level of inoculum (10^{-3} dilution) showed a significant difference between the different varieties at 21 days post-inoculation (P = 0.002). The group indistinguishable from Samsun included BinB5, D1, E1, G1, L3, P1, P2, P3, Q1, Q3, R1, S5, and W1. Plants that developed less disease symptoms were rated as B1 ≥ X5 ≥ B3.

Table 25. Analysis of symptoms observed in the inoculated tobacco plants 21 days p-i. The lower panel shows the Mean, Standard Deviation (95% confidence interval) derived from summed scores of symptoms per treatment.

Test 4 - Agro-infection with 10^{-4} dilution

Level	N	Mean	StDev
B1	3	1.6667	0.5774
B2	3	2.0000	0.0000
B3	3	0.6667	0.2887
Bin B5	3	2.0000	0.0000
D1	3	1.1667	1.6073
E1	3	2.6667	0.5774
G1	3	1.0000	1.7321
L3	3	2.0000	1.0000
P1	3	1.6667	1.1547
P2	3	0.6667	1.1547
P3	3	1.6667	0.5774
Q1	3	1.8333	1.2583
Q3	3	0.6667	1.1547
R1	3	0.0000	0.0000
S5	3	2.1667	1.4434
Samsun	3	2.0000	0.0000
W1	3	1.6667	0.5774
X5	3	0.3333	0.2887

Pooled StDev = 0.9354

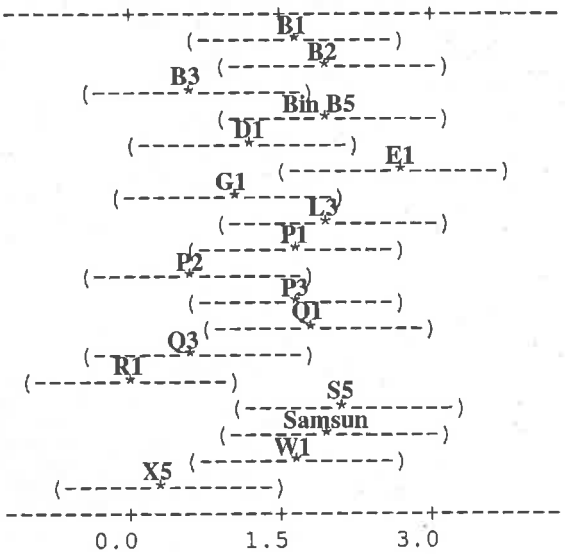
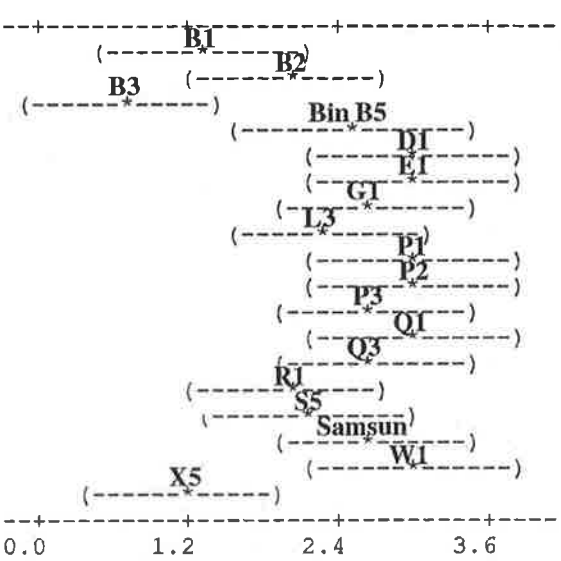


Table 26. Analysis of symptoms observed in the inoculated tobacco plants 21 days p-I with a 10-fold higher level of inoculum than shown in Table 25. The lower panel shows the Mean, Standard Deviation (95% confidence interval) derived from summed scores of symptoms per treatment.

Test 4 - Agro-infection with 10^{-3} dilution

Level	N	Mean	StDev
B1	3	1.3333	1.1547
B2	3	2.0000	0.0000
B3	3	0.6667	1.1547
Bin B5	2	2.5000	0.7071
D1	3	3.0000	0.0000
E1	3	3.0000	0.0000
G1	3	2.6667	0.5774
L3	3	2.3333	0.5774
P1	3	3.0000	0.0000
P2	3	3.0000	0.0000
P3	3	2.6667	0.5774
Q1	3	3.0000	0.0000
Q3	3	2.6667	0.5774
R1	3	2.0000	1.0000
S5	3	2.1667	1.4434
Samsun	3	2.6667	0.5774
W1	3	3.0000	0.0000
X5	3	1.1667	0.7638

Pooled StDev = 0.6866



In Test 5, the transgenic T_1 tobacco plants evaluated for disease symptoms 21 days after inoculation (Table 27) showed a significant variety effect ($P = < 0.001$) due primarily to less severe symptoms expressed in the X5 lines relative to Samsun (Table 28). The B3 line also exhibited less severe symptoms although the statistical analysis does not allow its complete separation from the main cluster of other transformed lincs. The symptoms of plants were rated as {Samsun, P2, Bin B5, R1, G1, D1, Q3, S5, B1, B3} ≥ X5.

The transgenic lines compared in Tests 7 and 8 included kanamycin-selected seedlings from the T_2 generation of the R1 transgenic tobacco line (Tables 27 and 28). The progeny of this line also maintained tolerance to challenge inoculation with ToLCV by displaying a delayed onset of disease symptoms similar to the B3 line. These analyses clearly separate these transgenic lines from the Samsun, transgenic Bin B5 and D1 transgenic tobacco lines.

Table 27. Summary of the statistical evaluation of differences between transgenic lines challenge-inoculated with ToLCV-DNA.

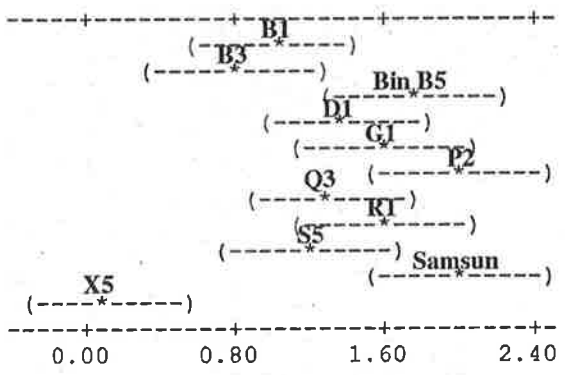
Test	Inoculum	Post-inoculation	*Probability (P)*	Response
5	10^{-4}	Day 21	< 0.001	{Samsun, P2, Bin B5, R1, G1, D1, Q3, S5, B1, B3} ≥ X5
6	10^{-4}	Day 21	< 0.001	{Samsun, Bin B5, D1} ≥ {R1A-(T_2), R1}
7	10^{-4}	Day 21	< 0.001	Samsun ≥ B1 ≥ R1A-(T_2) ≥ B3

Table 28. The analysis of the Means, Standard Deviation (95% confidence interval) derived from summed scores of symptoms per treatment in tests 6-8 tests are shown for 21 days post-inoculation.

Test 5. Agro-infection with 10^{-4} dilution

Level	N	Mean	StDev
B1	5	1.0000	0.7071
B3	5	0.8000	0.6708
Bin B5	4	1.7500	0.5000
D 1	5	1.4000	0.5477
G 1	5	1.6000	0.5477
P2	5	2.0000	0.0000
Q3	5	1.3000	0.6708
R1	5	1.6000	0.5477
S5	5	1.2000	0.4472
Samsun	5	2.0000	0.0000
X5	5	0.1000	0.2236

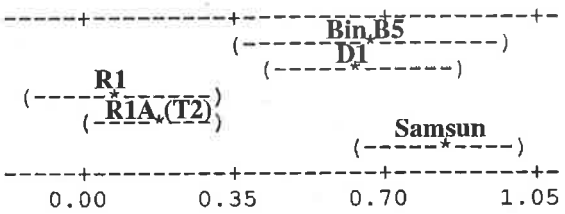
Pooled StDev = 0.5046



Test 6. Agro-infection with 10^{-4} dilution

Level	N	Mean	StDev
Bin B5	6	0.6667	0.5164
D1	14	0.6429	0.4972
R1	12	0.0833	0.2887
R1A (T2)	24	0.1667	0.3807
Samsun	18	0.8333	0.3835

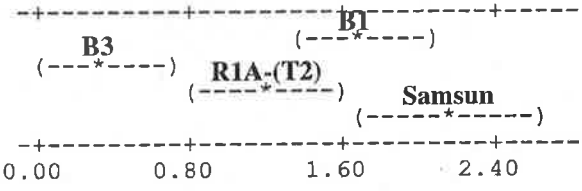
Pooled StDev = 0.4046



Test 7. Agro-infection with 10^{-4} dilution

Level	N	Mean	StDev
B1	10	1.7000	0.4830
B3	10	0.3500	0.4116
R1A-(T2)	9	1.1667	0.7071
Samsun	6	2.1667	0.7528

Pooled StDev = 0.5808



In Test 8, a further comparison was made between kanamycin-selected T_1 progeny of 35*S-c4* transgenic lines (X5, B3) and untransformed Samsun plants following challenge agro-infection with ToLCV-DNA inoculum (diluted to 10^{-4}). In addition, the segregating seedling population of the B3 line were separated into two groups, symptomless (B3N) and moderately abnormal (B3A), as shown in Figure 44. Although the X5 line also segregated into two groups with either severe or moderate foliar abnormalities, these were treated as a single population. Two plants of each treatment were left as the un-inoculated controls to indicate the relative stunting of the inoculated plants within each treatment.

All of the inoculated untransformed Samsun plants developed symptoms by day 12 following inoculation and progressively produced more severe symptoms with time. By comparison, symptoms induced in the 35*S-c4* transgenic lines did not occur until 20 days post-inoculation. By day 24, all of the transformed plants showed either very mild (0.5) or mild (1) disease symptoms compared with moderate (2) and severe symptoms (3) in the control Samsun plants. The foliar observations of the inoculated plants are summarised in Table 29.

Representative plants of each plant treatment at 30 days post-inoculation are shown in Figure 56. The inoculated plants of each group are shown by numbers 1 to 5 and reveal the stunted growth habit induced by the viral infection relative to the uninfected control plants (C). The inoculated B3N plants were less stunted than similarly treated plants in the other treatments. This may be explained, if the vegetative growth of B3A and X5 plants were penalised through interaction of the replicating viral DNA with lowered photosynthetic rates in the plants producing systemic yellow mosaic symptoms.

Table 29. The severity of viral symptoms displayed by plants inoculated with a 10^{-4} diluted ToLCV-DNA/*Agrobacterium* culture (**Test 8**).

Variety	12 days	16 days	20 days	24 days
Samsun	1, 1, 0.5, 0.5, 0.5, 0.5, 0.5, 0.5, 0.5, 0.5	2, 2, 1, 1, 1, 1, 0.5, 0.5, 0.5, 0.5	2, 2, 2, 2, 2, 2, 2, 2, 2, 2	3, 3, 3, 2, 2, 3, 2, 2, 2, 2
X5	0, 0, 0, 0, 0, 0, 0, 0, 0, 0,	0, 0, 0, 0, 0, 0, 0, 0, 0, 0	0, 0, 0.5, 0, 0, 0, 0, 0.5, 0.5, 0.5	0.5, 1, 1, 0.5, 1, 0.5, 0.5, 1, 1, 1
B3A	0, 0, 0, 0, 0, 0, 0, 0, 0, 0	0, 0, 0, 0, 0, 0, 0, 0, 0, 0	0.5, 0.5, 0, 0.5, 0, 0.5, 0.5, 0.5, 0.5, 0.5	1, 1, 1, 1, 0.5, 1, 1, 1, 1, 0.5
B3N	0, 0, 0, 0, 0, 0, 0, 0, 0, 0	0, 0, 0, 0, 0, 0, 0, 0, 0, 0	0.5, 0.5, 0.5, 0.5, 0, 0, 0.5, 0, 0.5, 0.5	0.5, 0.5, 0.5, 1, 0.5, 0.5, 1, 0.5, 1, 0.5

The dot-blot hybridisation of plant tissues sampled sequentially from the agro-inoculated plants at 12, 16, 20 and 24 days p-i is shown in Figure 54. Only one-half of the plants were sampled for dot-blot hybridisation on days 12, 16 and 20, whereas all of the plants were sampled on day 24. The plants sampled at a four-day interval did not appear to vary from plants sampled only once at day 24, as shown by the second row of radioactively-labelled DNA dots in each treatment. A summary of counts per minute (CPM) recorded for each dot sample is shown in Table 30.

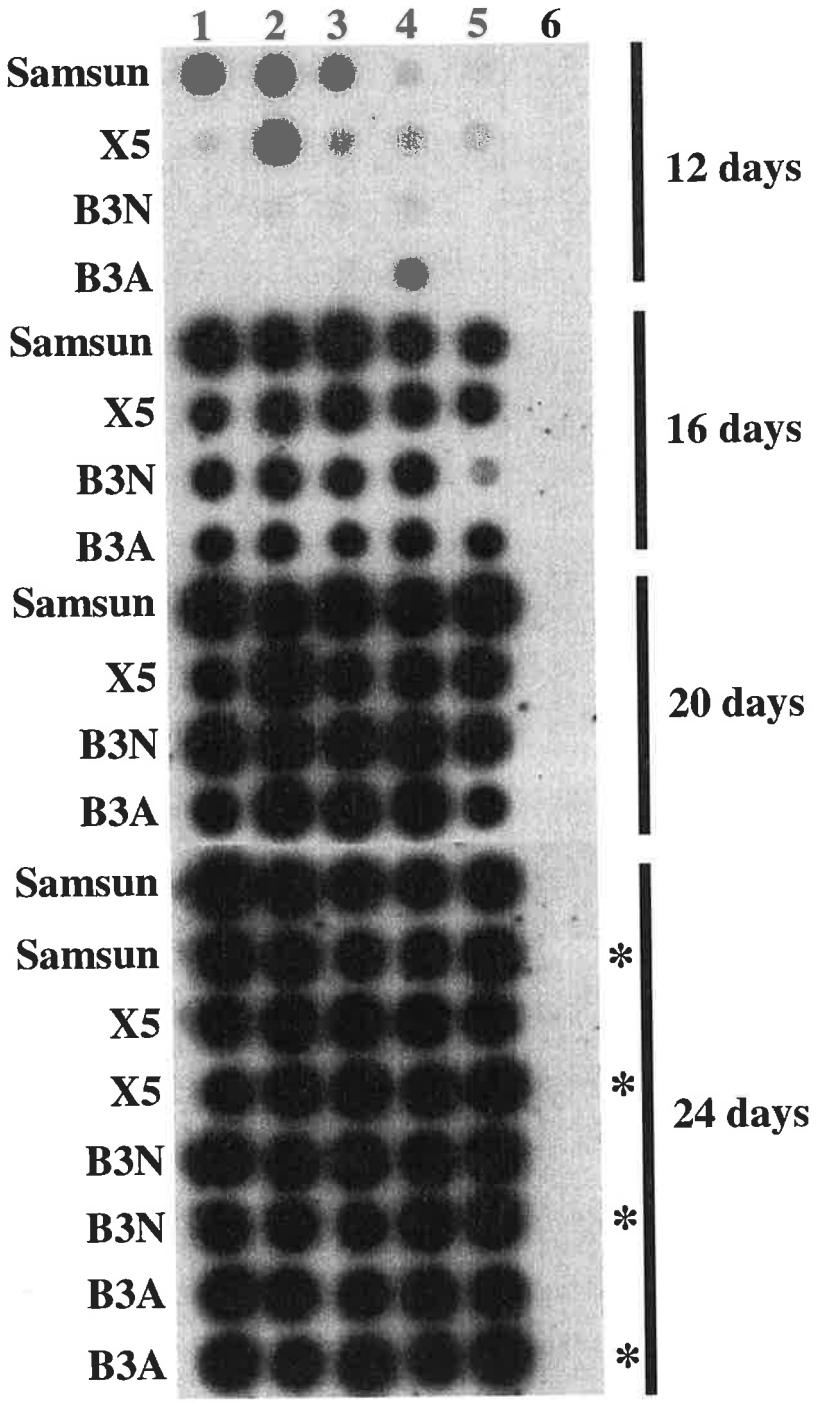


Figure 54. Dot-blot hybridisation of viral DNA in tissues of young leaves of plants infected with a 10^{-4} diluted ToLCV-DNA/*Agrobacterium* culture. The plants sampled only on day 24 post-inoculation are marked with an asterisk. An extract of the uninoculated control plant of each tobacco line was loaded in lane 6.

Table 30. Counts per minute (CPM) of radioactively-labelled DNA indicating the relative level of viral DNA in the inoculated plants.

Variety	CPM 12 days	CPM 16 days	CPM 20 days	CPM 24 days
Samsun- 1	145.9	879.4	1937.4	2256.5
Samsun- 2	138.4	717.1	1335.8	1357.3
Samsun- 3	93.7	995.3	1785	1113.8
Samsun- 4	53.9	368	1303.3	1014.5
Samsun- 5	45.9	326.7	2054	1204.6
Samsun- 6 - uninoculated	39.8	42.6	41	45.6
Samsun- 7				1315.4
Samsun- 8				1084.9
Samsun- 9				834.3
Samsun- 10				783.1
Samsun- 11				1501.8
Samsun-12 - uninoculated				42.8
X5- 1	45.9	223.3	456.3	903.2
X5- 2	175.5	393.8	2829.9	1451.9
X5- 3	65.8	449.8	676.6	1387.5
X5- 4	66.8	335.7	787.1	1103.7
X5- 5	54.3	190.2	806.2	1116.2
X5-6 - uninoculated	41.8	51.6	42.3	43
X5- 7				614.8
X5- 8				1194.1
X5- 9				1660.5
X5- 10				1256.3
X5- 11				1689.5
X5- 12-uninoculated				42
B3N- 1	43	230.3	1675	1263.2
B3N- 2	46.7	287	1706.9	1159.7
B3N- 3	46.4	208.2	1466.1	1420.5
B3N- 4	48.3	235.4	1870.5	1138.1
B3N- 5	43.4	60.8	706.9	1331.4
B3N-6 - uninoculated	38.5	43.7	43.6	38
B3N- 7				876.4
B3N- 8				887.8
B3N- 9				652.2
B3N- 10				1176.2
B3N- 11				1362.4
B3N-12 - uninoculated				39.3
B3A- 1	40.9	185	437.5	1317.5
B3A- 2	38.9	189.1	1499.8	1131.6
B3A- 3	48.3	141.1	1464.2	785.8
B3A- 4	79.4	226.4	1217	1090.2
B3A- 5	40.9	169.7	257.1	950
B3A-6 - uninoculated	42	42.5	40.8	43
B3A- 7				1288.3
B3A- 8				775.4
B3A- 9				1295.2
B3A- 10				866.8
B3A- 11				1331.9
B3A-12 - uninoculated				42.6

Initial evaluation of the CPM data set indicated that a logarithmic transformation was required to reduce variability. This resulted in standardised residuals that were more normally distributed. The analysis of variance of the log-transformed data is given in Table 31. There were large effects due to day and variety with a significant day by variety effect. The Samsun plants generally had the higher values and thus a higher level of viral DNA than all other lines. Statistically significant differences between the transgenic lines and the Samsun was reached at day 16, post-inoculation (Figure 55). However by day 24, the differences between the varieties were smaller and not statistically significant.

Table 31. Analysis of variance for CPM for data set shown in Table 30 after log transformation. Variate: logCPM.

Source	DF	SS	MS	F	*Probability*
Variety	3	0.9743	0.32477	11.51	< 0.001
Day	3	22.52981	7.50994	266.06	< 0.001
Variety x Day	9	0.59653	0.06628	2.35	0.024
Residual	60	1.69362	0.02823		
Total	79	26.33297			

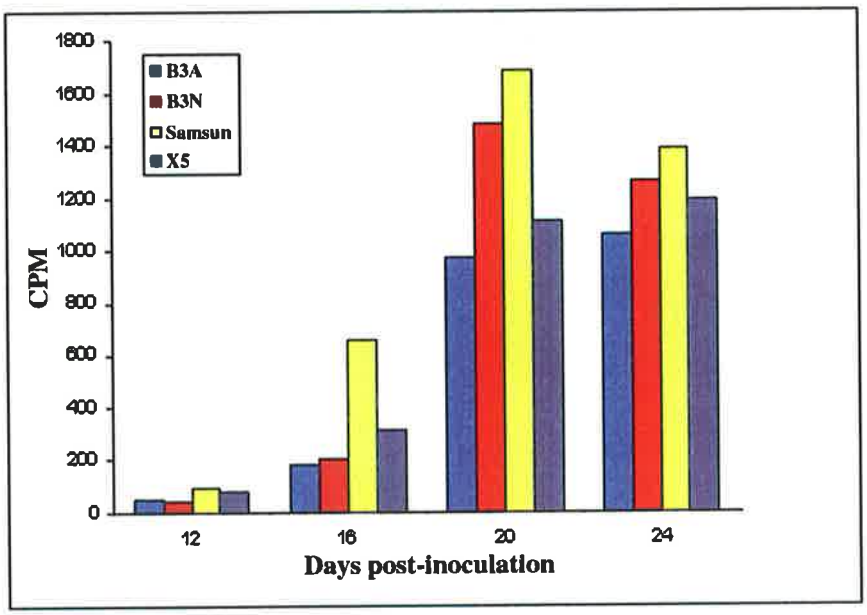


Figure 55. Comparison of the level of viral DNA as indicated by the mean CPM of dot-blots of plant tissue sampled from the virus infected plants on day 12 to 24 post-inoculation.

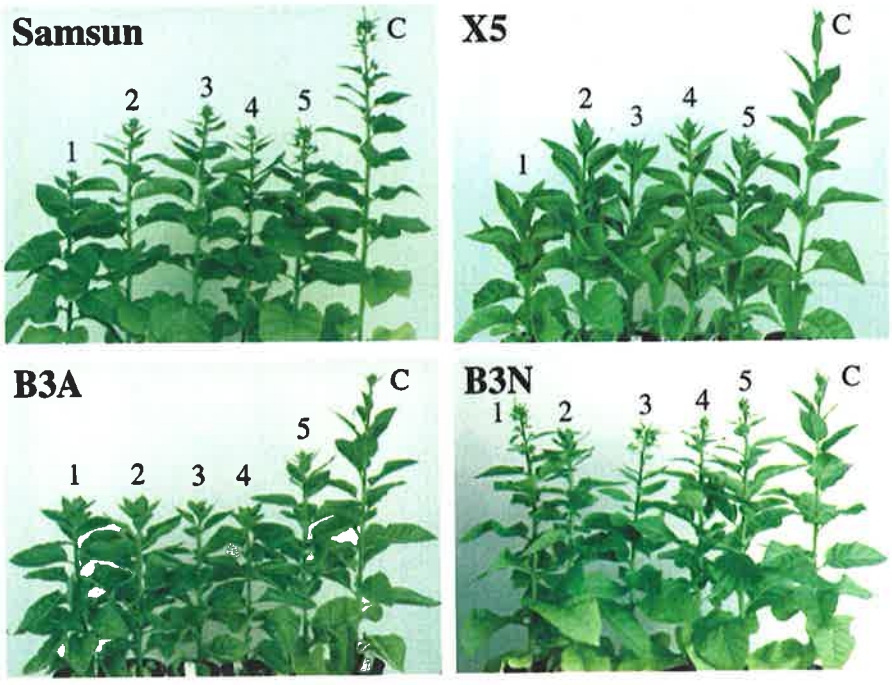


Figure 56. A comparison of the group of five agro-inoculated plants of Samsun with kanamycin-selected T_1 transgenic lines (X5, B3A, B3N). A non-inoculated control plant of each line is indicated by "C."

4.3.3 Agro-inoculation of 35*S-c4 f-s* transgenic tobacco

Forty-four kanamycin-selected 35*S-c4 f-s* transgenic tobacco lines of the T_0 generation were agro-inoculated with a 10^{-4} diluted ToLCV-DNA/*Agrobacterium* culture. None of these lines were immune to infection although 45% of them displayed delayed onset of induced viral symptoms by approximately 7 days or longer compared with the inoculated Samsun plants. Although this observation was consistent with effects induced in transgenic 35*S-c4* plants, the 35*S-c4 f-s* plants were older and longer established in their containers compared with the younger Samsun control plants at the time of inoculation. No attempt was made to further test the T_1 progeny of selected 35*S-c4 f-s* tobacco lines under more controlled conditions as transgenic tomato plants had been regenerated with the same gene construction and were available for testing (**4.3.3**).

4.3.4 Agro-inoculation of 35*S-c4* and 35*S-c4 f-s* transformed tomato

The disease symptoms induced in the Ailsa Craig variety of tomato following challenge agro-inoculation with ToLCV were identical to those of the Gross Lisse variety of tomato illustrated in Figure 17 (**1.8.2.4**). Likewise, the susceptible transgenic 35*S-c4* and 35*S-c4 f-s* lines also displayed the same foliar symptoms following infection. None of the severely abnormal plants derived from the 35*S-c4* cultures were tested for resistance to viral infection. Instead, the focus was shifted towards assessing only lines with normal vegetative growth. The initial test of transformed 35*S c4 f-s* lines with the 10^{-4} diluted inoculum indicated a mixture of phenotypes ranging from susceptible to apparently resistant to agro-infection with ToLCV-DNA (Table 32). These were evaluated visually for the presence of induced virus disease symptoms (Figure 57) and by dot-blot hybridisation with a specific viral probe (Figure 58).

The initial screening of the T_0 generation of kanamycin-resistant plants derived from the 35*S-c4 f-s* cultures indicated that three lines of *c4 f-s* (1-7, 4-4, 5-5) displayed potential resistance to viral infection (Table 32). Young plants derived from rooted cuttings were obtained from each parental plant of *c4 f-s* 1-7, 4-4, and 5-5, to provide replicates of the T_0 generation for further assessment of resistance to challenge-agro-inoculation with ToLCV-DNA (Tables 33 and 34). These plants showed lower rates of disease symptoms than the corresponding Ailsa Craig control plants in the respective tests, although no uniform immunity of any line was observed.

Table 32. Response of the 35*S c4 f-s* transformed tomato (T_0) and the untransformed Ailsa Craig plants evaluated 40 days after inoculation following infection with a 10^{-4} diluted ToLCV-DNA/*Agrobacterium* culture. The severity of symptoms (0–3) displayed by individual plants is shown in parenthesis.

Observed response	Severity of induced symptoms
Susceptible	Ailsa Craig (3,3,3,3,3,3,3,3,3,3) *c4 f-s* 1-1 (2) *c4 f-s* 1-2 (3) *c4 f-s* 4-5 (3) *c4 f-s* 5-6 (3) *c4 f-s* 6-1 (2) *c4 f-s* 6-2 (3) *c4 f-s* 6-4 (3)
Tolerant	*c4 f-s* 4-2 (1) *c4 f-s* 4-3 (1) *c4 f-s* 5-1 (1)
No infection detected	*c4 f-s* 1-7 (0) *c4 f-s* 4-4 (0) *c4 f-s* 5-5 (0)

Figure 57. Comparison of selected T_0 generation of 35*S-c4 f-s* transgenic tomato lines displaying susceptibility (left), tolerance (centre) and apparent resistance (right) to viral infection by inoculation with a 10^{-4} diluted ToLCV-DNA/*Agrobacterium* culture.

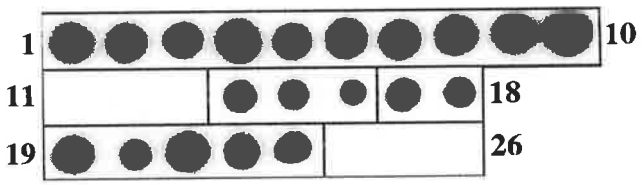


Figure 58. Dot-blot hybridisation of tomato leaf tissue samples from the ToLCV-DNA agro-infected tomato plants referred to in Table 32. DNA was extracted from the infected untransformed Ailsa Craig plants (spots 1 - 10); *c4 f-s* lines 1-7, 4-4, 5-5 with apparent resistance to viral infection (spots 11 - 13); *c4 f-s* lines 4-2, 4-3, 5-1 with mild symptoms (spots 14 – 16); *c4 f-s* lines 1-1, 6-1 with moderate viral disease symptoms (spots 17 – 18); *c4 f-s* lines 1-2, 4-5, 5-6, 6-2, 6-4 with severe viral symptoms (spots 19 – 23) and uninoculated Ailsa Craig plants (spots 24 – 26). The cDNA probe used was made from a full-length ToLCV-DNA template described in **2.5.11.6** and **2.5.11.7.**

The differences between the *c4 f-s* 1-7, 4-4, and 5-5 lines and control Ailsa Craig plants appeared when a lower concentration of inoculum (10^{-4}) was used but these differences were insignificant when a 10-fold the higher level of inoculum (10^{-3}) was used. This indicated a dose related effect on the ability of the selected transformed lines to resist ToLCV infection (Tables 33 and 34). Analysis of the agro-inoculated plants in Tests 13 and 14 for viral DNA replication is shown in Figure 59. The presence of viral DNA in the extracts of plants matched the infection rate shown in Table 33. The level of hybridised viral probe bound to spots of DNA from the transgenic plants tested, reached the intensity equivalent to that of DNA from the infected wild-type plants in only 3 of 34 samples.

Table 33. Rates of virus disease induced in the vegetatively propagated T_0 generation transformed tomato lines and untransformed Ailsa Craig plants evaluated 40 days p-i following inoculation with a 10^{-4} diluted ToLCV-DNA/*Agrobacterium* culture. The treatments for direct comparison are indicated in parenthesis.

Variety	Numbers of plants symptomatic/ total plants inoculated in individual tests	Total	Average % diseased
Ailsa Craig	(9) 10/10, (10) 5/5, (11) 5/7, (13) 9/10, (14) 6/6, (15) 6/7, (16) 6/6, (17) 5/5	52/56	93%
C4 1-1	(10) 0/3		0%
C4 1-6	(10) 2/2		100%
C4 1-8	(10) 2/3		66%
C4 2-1	(10) 2/3		66%
C4 2-4	(10) 2/2		100%
C4 5-6	(10) 2/3		66%
C4 f-s 1-1	(9) 1/1		100%
C4 f-s 1-2	(9) 1/1		100%
C4 f-s 1-4	(10) 2/3		66%
C4 f-s 1-5	(10) 2/2		100%
C4 f-s 1-7	(9) 0/1, (10) 2/5, (11) 4/20, (13) 2/5, (14) 2/6, (16) 4/5, (17) 2/5,	16/48	34%
C4 f-s 2-1	(10) 3/4		75%
C4 f-s 2-2	(10) 3/3		100%
C4 f-s 3-1	(10) 0/2		0%
C4 f-s 4-2	(9) 1/1,		100%
C4 f-s 4-3	(9) 1/1,		100%
C4 f-s 4-4	(9) 0/1, (10) 0/7, (11) 5/15, (13) 0/7, (14) 0/8, (15) 5/9, (16) 3/7, (17) 0/7	13/61	21%
C4 f-s 4-5	(9) 1/1,		100%
C4 f-s 5-1	(9) 1/1,		100%
C4 f-s 5-5	(9) 0/1, (10) 0/3, (11) 2/9, (13) 0/3, (14) 0/4, (15) 3/7, (16) 1/5, (17) 0/3	6/35	17%
C4 f-s 5-6	(9) 1/1,		100%
C4 f-s 5-7	(10) 2/3		66%
C4 f-s 6-1	(9) 1/1,		100%
C4 f-s 6-2	(9) 1/1,		100%
C4 f-s 6-4	(10) 3/3		100%
C4 f-s 6-5	(10) 3/3		100%
C4 f-s 6-6	(9) 1/1		100%

Table 34. Rates of virus disease induced in the vegetatively propagated T_0 generation transformed tomato lines and untransformed Ailsa Craig plants evaluated 40 days p-i following inoculation with a 10^{-4} diluted ToLCV-DNA/*Agrobacterium* culture. The treatments for direct comparison are indicated in parenthesis.

Variety	Infected/total plants inoculated in the individual tests	Total	% diseased
Ailsa Craig	(12) 11/12, (15) 4/4	15/16	94%
C4 f-s 1-7	(12) 5/11, (15) 6/6	11/17	65%
C4 f-s 4-4	(15) 6/7		86%
C4 f-s 5-5	(15) 4/5		80%

Logistic regression analysis on the binomial data derived from Table 33 was used to assess the statistical significance of the relative proportions of viral infection induced in the vegetatively replicated Ailsa Craig versus replicates from three 35*S c4 f-s* lines (1-7, 4-4 and 5-5). The data set showing the proportions of infection for each of the eight tomato tests are tabulated in Table 35 and displayed in Figure 60. The statistical analysis showed that there was a highly significant varieties effect due to the transgenic lines (P <0.001) and a smaller but significant difference between the individual tests (P <0.05). The significant varieties effect was partitioned into orthogonal comparisons to test for differences between Ailsa Craig and the transformed lines and between the transformed lines.

A highly significant difference was obtained by orthogonal comparison of the inoculated Ailsa Craig variety and the inoculated three transformed tomato lines (P <0.001). Further comparison showed that the inoculated 1-7 line differed from the inoculated 4-4 and 5-5 lines (P <0.01) and a non-significant orthogonal comparison (P >0.378) was obtained between the inoculated 4-4 and 5-5 lines.

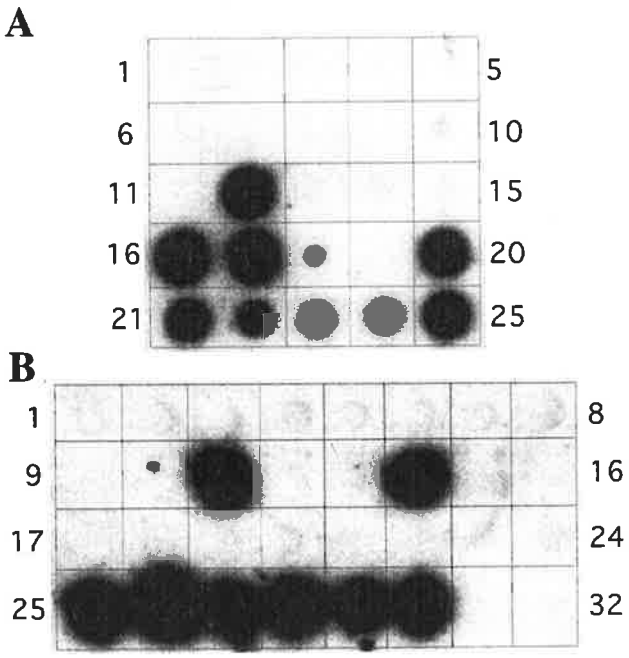


Figure 59. Dot-blot hybridisation of tomato leaf tissue samples from T_0 generation tomato replicates in Tests 13 (A) and 14 (B) that were infected with a 10^{-4} diluted ToLCV-DNA/*Agrobacterium* culture. The cDNA probe used was made from a full-length ToLCV-DNA template described in **2.5.11.6** and **2.5.11.7.**

In panel A, the DNA was extracted from:

c4 f-s 4-4 (spots 1 – 7)

c4 f-s 1-7 (spots 8 – 12)

c4 f-s 5-5 (spots 13 – 15)

untransformed Ailsa Craig (spots 16 - 25)

In panel B, the DNA was extracted from:

c4 f-s 4-4 (spots 1 - 8)

c4 f-s 1-7 (spots 9 - 15)

c4 f-s 5-5 (spots 17 – 20)

untransformed Ailsa Craig (spots 25 – 30)

uninoculated healthy Ailsa Craig (spots 22 – 24)

Positions at 16 and 31 – 32 were left blank.

Table 35. The proportions of disease symptoms induced in the vegetative replicates of Ailsa Craig tomato and replicates of three transformed T_0 generation lines (1-7, 4-4, 5-5) following inoculation with a 10^{-4} diluted ToLCV-DNA/*Agrobacterium* culture.

Test	Ailsa Craig	*c4 f-s* 1-7	*c4 f-s* 4-4	*c4 f-s* 5-5	Overall
9	1.00	0.00	0.00	0.00	0.77
10	1.00	0.40	0.00	0.00	0.35
11	0.71	0.20	0.33	0.22	0.32
13	0.90	0.40	0.00	0.00	0.44
14	1.00	0.33	0.00	0.00	0.33
15	0.86		0.55	0.43	0.61
16	1.00	0.80	0.43	0.20	0.61
17	1.00	0.40	0.00	0.00	0.35
Overall	0.93	0.34	0.21	0.17	0.44
Ranking	a	b	c	c	(P <0.001)

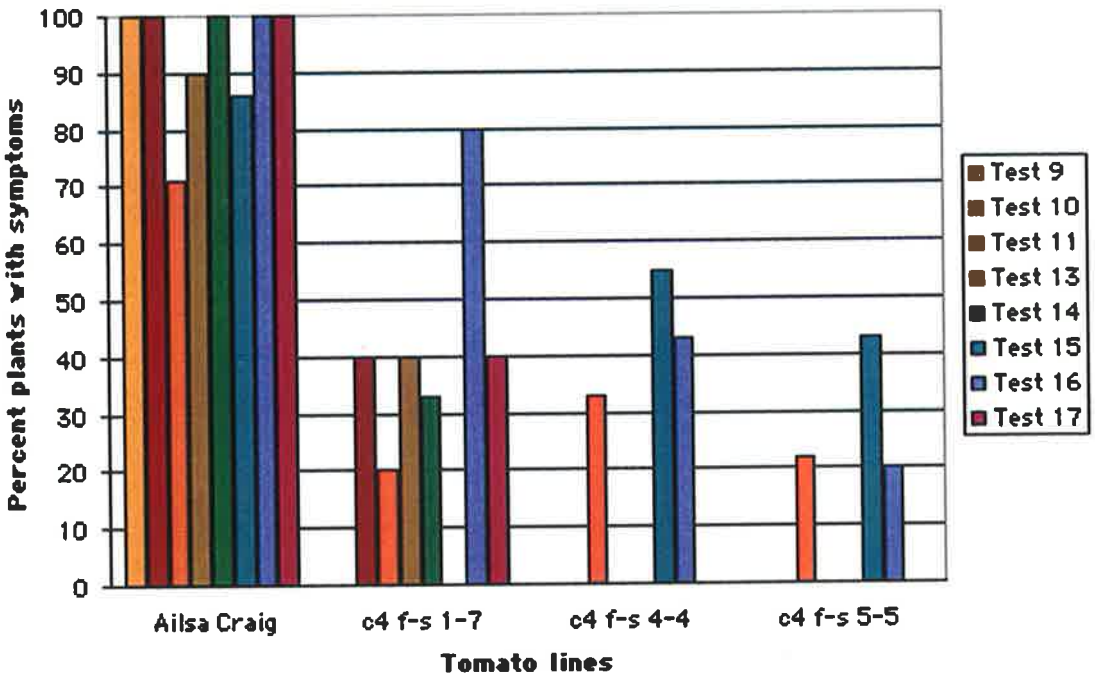


Figure 60. The percentage of virus diseased plants of untransformed Ailsa Craig and three transformed *c4 f-s* lines (1-7, 4-4 and 5-5) of the T_0 generation evaluated 40 days after infection with a 10^{-4} diluted ToLCV-DNA/*Agrobacterium* culture.

Attempts to select a segregating population of plants from the T_1 generation of the *c4 f-s* 1-7, 4-4 and 5-5 seedlings using kanamycin selection at either 50 μg ml^{-1} or 100 μg ml^{-1} were unsuccessful. At the lower concentration, the efficient selection for kanamycin resistance *in vitro* was not achieved. Selection at the higher concentration of kanamycin produced stunting of all plants that persisted even when plants were moved onto *in vitro* media without kanamycin, or into potting media in a glasshouse. Intermediate concentrations of kanamycin were not evaluated. Instead, an unselected population of seedlings from each plant line were grown under conditions identical to the Ailsa Craig seedlings. All the seedling plants were agro-inoculated at the six to eight leaf stage, as described in **5.2.1**. The visible response of plants to agro-infection with a 10^{-4} or 10^{-3} inoculum is summarised in Table 36.

Table 36. Rates of virus disease symptoms induced in the unselected T_1 generation seedlings of 35*S c4-f-s* transgenic tomato lines and Ailsa Craig plants following agro-inoculation with ToLCV-DNA. The treatments for direct comparison within individual tests are indicated in parenthesis.

Agro-infection with 10^{-4} dilution – evaluated 40 days p-i

Variety	Numbers of plants symptomatic/total plants inoculated in individual tests	Total	% diseased
Ailsa Craig	(18) 10/20, (19) 18/20, (20) 14/20	42/60	70%
C4 f-s 1-7	(18) 0/20, (19) 5/20, (20) 5/22	10/62	16%
C4 f-s 4-4	(18) 5/20, (19) 10/20, (20) 8/22	23/62	37%
C4 f-s 5-5	(18) 3/14, (19) 7/15, (20) 5/22	15/51	29%

Agro-infection with 10^{-3} dilution – evaluated 40 days p-i

Variety	Numbers of plants symptomatic/total plants inoculated	% diseased
Ailsa Craig	(18) 18/18	100%
C4 f-s 1-7	(18) 19/20	95%
C4 f-s 4-4	(18) 19/20	95%
C4 f-s 5-5	(18) 13/13	100%

The transformed tomato plants agro-inoculated with the lower level of inoculum appeared to show lower rates of disease symptoms than the similarly inoculated untransformed Ailsa Craig plants. The tabulated proportions of virus disease from each treatment are shown in Table 37 and Figure 61.

Statistical analysis of the data set in Table 37 with a logistic regression method showed a highly significant varieties effect (P <0.001) and a significant difference between the individual tests (P <0.01). The orthogonal comparisons showed that the inoculated Alisa Craig plants were significantly more susceptible to ToLCV infection than the three transformed tomato lines (P <0.001). After removal of the effect of Ailsa Craig, there remained a significant varieties effect (P <0.05). Subsequent orthogonal comparisons revealed a significant difference (P <0.01) between the *c4 f-s* 1-7 line and the *c4 f-s* 4-4 and 5-5 lines but a non-significant orthogonal comparison between the 4-4 and 5-5 lines (P >0.356). The difference between the treatments disappeared when the plants were agro-inoculated with a 10-fold higher level (10^{-3} dilution) of ToLCV-DNA inoculum (Table 37).

Table 37. The proportions of viral disease induced in the seedlings of Ailsa Craig tomato and three transformed T_1 generation lines (1-7, 4-4, 5-5) evaluated 40 days after infection with a10^{-4} diluted ToLCV-DNA/*Agrobacterium* culture.

Test	Ailsa Craig	*c4 f-s* 1-7	*c4 f-s* 4-4	*c4 f-s* 5-5	Overall
18	0.50	0.00	0.25	0.21	0.24
19	0.90	0.25	0.50	0.47	0.53
20	0.70	0.23	0.36	0.23	0.37
Overall	0.70	0.16	0.37	0.29	0.38
Ranking	a	b	c	c	(P <0.001)

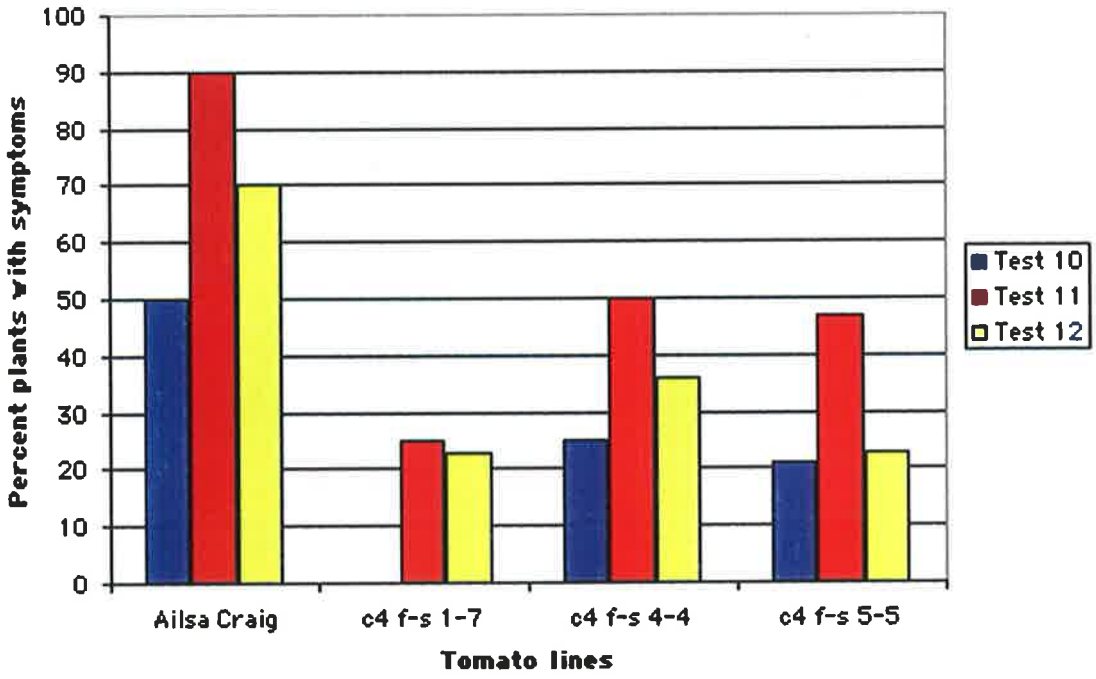


Figure 61. The percentage of virus diseased plants of untransformed Ailsa Craig and three transformed *c4 f-s* lines (1-7, 4-4 and 5-5) of the T_1 generation evaluated 40 days following infection with a 10^{-4} diluted ToLCV-DNA/*Agrobacterium* culture.

Total nucleic acid was extracted from young leaves of the inoculated plants 28 days p-i to determine the presence of viral DNA by dot-blot hybridisation (Figure 62). The detection of viral DNA in the inoculatcd Ailsa Craig plants agreed with the record of visual symptoms, 85% of plants with viral DNA compared with 90% plants that developed symptoms in Test 19 and equal percentages of viral DNA and disease symptoms in Test 20. In contrast, the inoculated transformed tomato lines showed a delay in the development of symptoms compared with the replication of viral DNA in the same plants. In Test 19, the difference between the percentage of plants that produced viral DNA and those that showed symptoms was +10% for the *c4 f-s* 1-7 line, +5% for the *c4 f-s* 4-4 line and +17% for the *c4 f-s* 5-5 line. The difference was higher in Test 20 with +17% for the *c4 f-s* 1-7 line, 28% and 27% for the *c4 f-s* 4-4 and 5-5

lines, respectively. These results are consistent with observations made with the inoculated 35*S*-*c4* transformed tobacco plants (**4.3.2**).

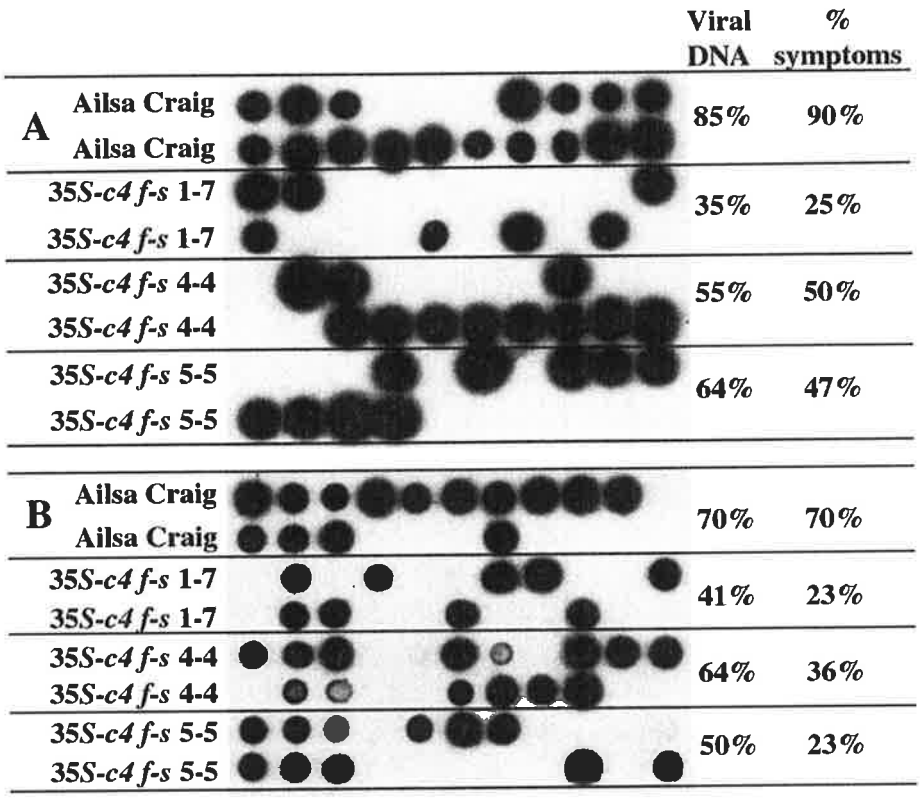


Figure 62. Dot-blot hybridisation of extracts of tomato leaf tissue sampled from ToLCV-DNA inoculated Ailsa Craig and unselected T_1 generation seedlings from tomato lines transformed with a 35*S*-*c4 f-s* gene construction shown in Test 19 (A) and Test 20 (B). The percentage of diseased plants at 28 days p-i is shown for each treatment. The cDNA probe used was made from a full-length ToLCV-DNA template described in **2.5.11.6** and **2.5.11.7.**

Although the *c4 f-s* 1-7, 4-4 and 5-5 lines were all less susceptible to challenge-inoculation with ToLCV-DNA, only the 5-5 line was shown to express the *c4 f-s* transgene in the northern-blot analysis (Figure 41). Each of the three lines was tested for the presence of the *nptII* gene and for the *c4 f-s* insert by PCR. In the limited PCR testing that was done, the *nptII* gene was detected in the *c4 f-s* 4-4 and 5-5 tomato lines

(Figure 63A) but the presence of the *c4 f-s* insert was found only in the 5-5 line (Figure 63B). This indicated that the *c4 f-s* 4-4 and 1-7 lines, may not contain the complete transgene.

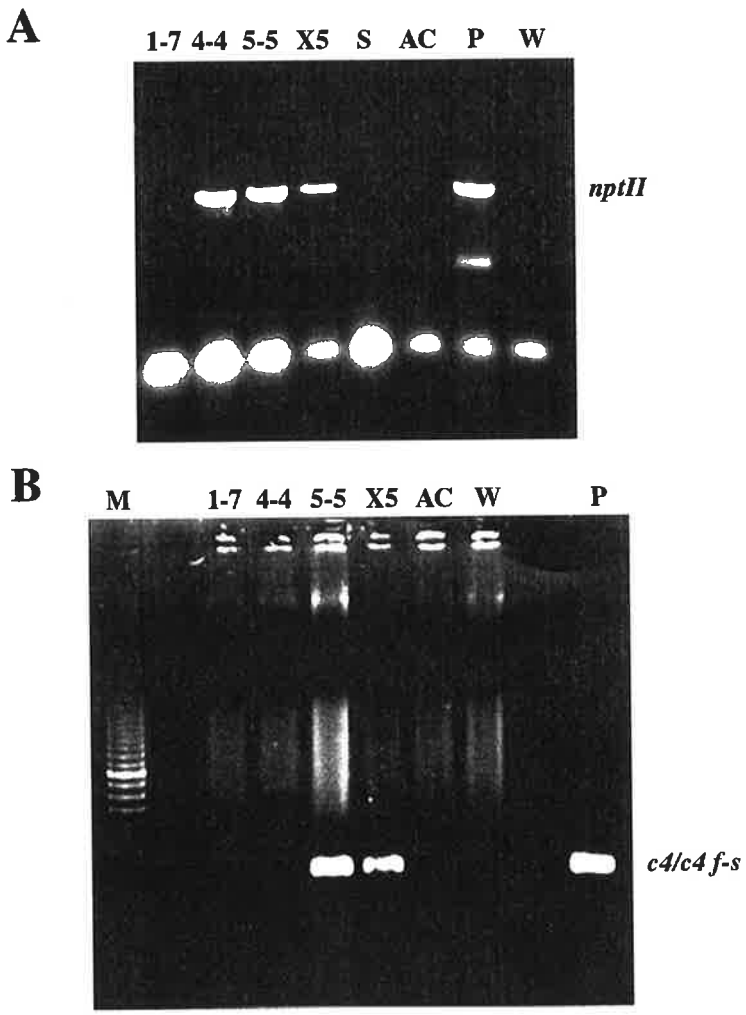


Figure 63. Detection of the *nptII* gene (A) and the *c4* and *c4 f-s* transgenes in the transformed 35*S c4 f-s* 1-7, 4-4 and 5-5 tomato lines by PCR. Other samples used for comparison included an extract of the X5 line of 35*S-c4* tobacco (X5), healthy Samsun tobacco (S), healthy Ailsa Craig tomato (AC), plasmid pBin*c4* (P) and a water/buffer control (W). The primers used and conditions for the PCR are described in sections **2.5.9.2** and **2.5.9.3**, respectively.

4.4 Discussion

The agro-infection method of Grimsley *et al.,* (1986) is an efficient means of infecting plants with viral DNA. This method has been commonly used to screen transgenic plants for resistance to geminiviruses (Grimsley *et al.*, 1987; Elmer *et al.,* 1988b; Stanley *et al.*, 1990; Day *et al.*, 1991; Stenger 1994; Michelson *et al.*, 1994; Noris *et al.*, 1996; Bendahmane and Gronenborn, 1997; Brunetti *et al.*, 1997). In this procedure, a greater than genome length of cloned DNA is inserted into the T-DNA of *Agrobacterium tumefaciens* and delivered by injection to the plants. Following integration of the viral insert into the host plant genome, a unit copy of the viral genome is released, undergoes replication and eventually spreads systemically through the vascular system of a susceptible host. As a result of agro-inoculation, the early steps in natural infection including insect vector-plant interaction, uncoating of the virions and synthesis of the DNA replicative intermediate form are by-passed, raising the possibility that agro-inoculation may not equate with whitefly-mediated transmission. In support of this, it was reported that agro-infection of wild *Lycopersicon* species with TYLCSV-DNA overcame the natural viral resistance displayed by these plant species against whitefly-mediated virus transmission (Kheyr-Pour *et al.*, 1994).

In this study, a 10 µl aliquot of a 10^{-4} dilution of a standardised *Agrobacterium*-ToLCV-DNA inoculum (0.56 x 10^{-4} OD_{600}) was found to generally produce a 100% infection rate of the inoculated Samsun tobacco and Ailsa Craig tomato plants (Table 19). This dose was found to contain 2,200 bacterial colonies when spread on plates and was consistent with the report by Elmer *et al.*, (1988b) that only 2,000 *Agrobacterium* cells could produce 100% infection rates in permissive transgenic plants. Under the test conditions used in this study, several of the transformed 35*S-c4* and 35*S-c4 f-s* tobacco

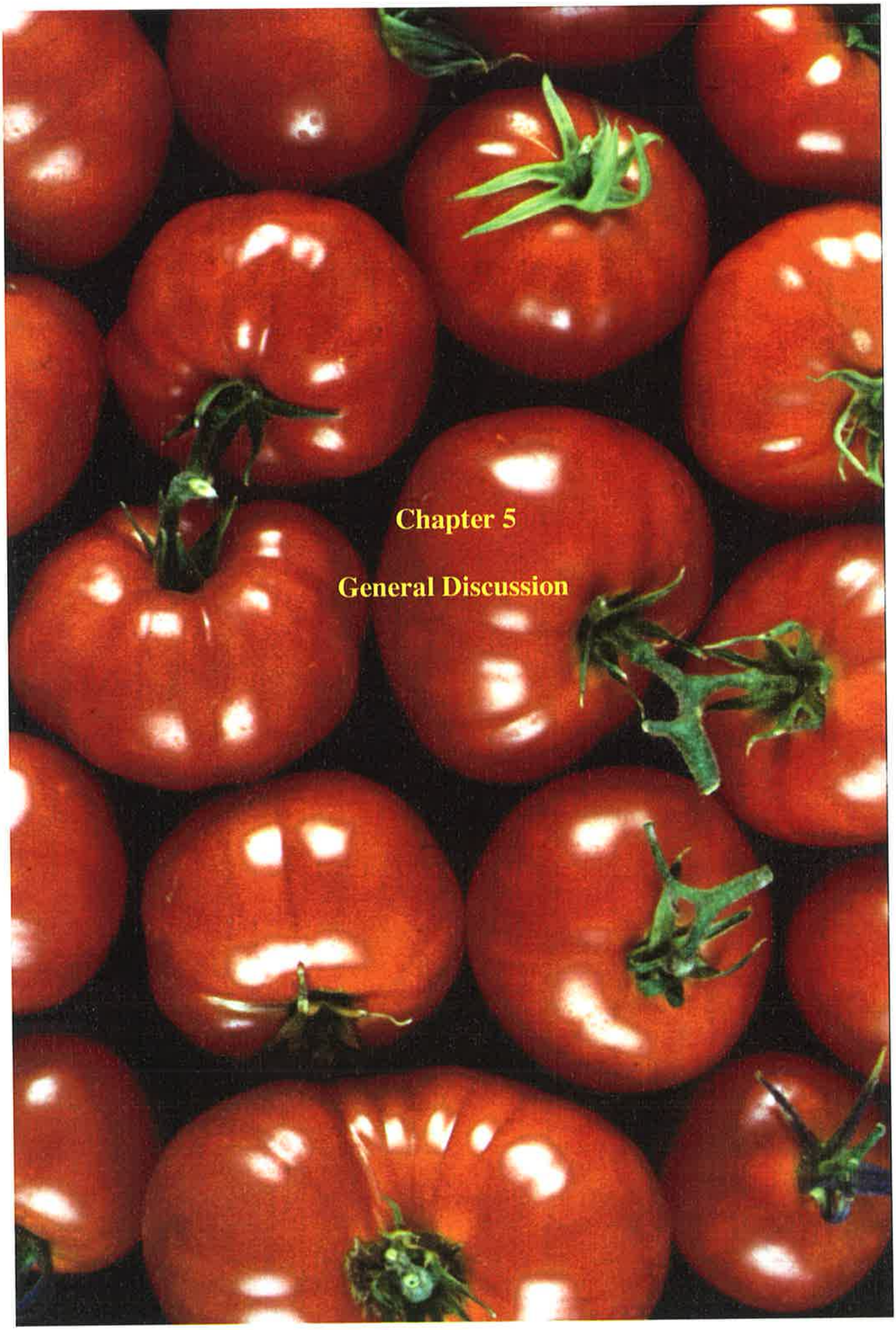
and tomato lines displayed the ability to resist or tolerate infection by ToLCV-DNA. These characteristics were demonstrated by a delay in the onset of viral disease symptoms, lower levels or absence of replicating viral DNA, development of less severe disease symptoms, and lower rates of infection in the inoculated transgenic plants compared with the respective control plants (Tables 20, 25-26, 29 and Figures 54-61). Also, some of the transformed tobacco and tomato plants were shown to have symptomless infections. These effects were statistically significant as shown in Tables 21, 26, 28, 30-32, 36 and 39. However, none of the transgenic tobacco and tomato plants tested were immune to infection when a higher inoculum level (10^{-3}) was used.

The challenge agro-inoculation of Samsun tobacco with ToLCV-DNA versus an inoculum of a mutated version of ToLCV-DNA with a dysfunctional C4 ORF reproduced the effects in the Samsun plants reported by Rigden et al. (1994). The inoculation of the 35*S-c4* transgenic lines with either ToLCV-DNA or the C4 mutated version of ToLCV-DNA gave some interesting effects. For instance, the P3 line appeared to be more susceptible to infection by ToLCV-DNA and the C4 mutant-DNA than the wild-type Samsun plants (Table 20). There was no indication of reversion of the viral mutant back to the natural ToLCV-DNA state as the rating of symptoms displayed by plants inoculated with the C4 mutant-DNA was correspondingly lower than those induced in plants inoculated with ToLCV-DNA. Also, some of the P3 plants inoculated with the C4 mutant-DNA began to show the initial signs of infection followed by apparent recovery from symptoms with subsequent re-expression of the disease symptoms. Recovery from the initial stage of infection was also observed in some plants of the X5 and B3 transgenic lines. Virologists have recognised this phenomenon as a form of natural resistance of individual plants against genotypic

variants of a given virus (Pennazio *et al.*, 1999). Agro-inoculation of the transformed lines with C4 mutant-DNA at a 10-fold higher inoculum dose (0.56×10^{-3}) indicated some ability of each of the 35*S-c4* lines to resist infection from the C4 mutant viral DNA (Tables 23 and 24).

Generally, the detection of ToLCV-DNA in the inoculated plants matched the record of disease symptoms displayed by these plants. However in some cases, individual T_1 progeny of the R1, B3, X5, Q3 and B1 lines which contained viral DNA did not show disease symptoms at a month after agro-infection (Table 25). This seemed to indicate that these plants had the ability to tolerate the infection of ToLCV, at least in the short term. In each of the challenge-inoculation tests with ToLCV-DNA, the X5, B3 and R1 lines consistently displayed lower rates of infection and delayed development of disease than the corresponding inoculated control Samsun plants (Tables 20, 25-26, 27, 30-32) and those derived from a transformed pBin19 line. The effect was also reproduced in the T_2 generation of the R1 transgenic line (Table 32). In many cases, the delay in disease development was in the order of seven days compared to control plants whereas in other cases it was much longer. The reason for this has not been determined but it appears unrelated to the number of gene inserts integrated into the plant genome (Table 16), or to the level of the transgene expression. For instance, kanamycin-selected T_1 generation plants of the X5 and P3 lines expressed a high level of the *c4* transgene, while plants of the B3 line expressed multiple bands of RNA species larger than the *c4* transcript whereas no *c4* RNA transcript was detected in plants of the R1 line (Figures 39 and 44).

Similarly, differences were observed between some of the 35*S c4 f-s* transformed lines of tomato that showed the ability to resist infection by ToLCV-DNA at a lower level of inoculum. The *c4 f-s* RNA transcript was detected in the 5-5 line (Figure 41) but not in either of the 1-7 and 4-4 lines that exhibited similar resistance characteristics (Tables 34 and 37, Figures 58-62). Although several transformed lines produced showed the ability to resist and tolerate challenge agro-inoculation by ToLCV-DNA, it remains to be demonstrated if any of these transformed lines have resistance to ToLCV via whitefly-mediated viral transmission.



Chapter 5

General Discussion

The cultivation of the tomato is an important economic crop for many countries of the World. In the year 2000, over 3.5 million hectares were devoted to its cultivation (FaoStat Database). The yearly average yield per hectare has been generally increasing in many countries (Figures 7-15) despite the reported increasing crop loss due to pathogens including the whitefly-transmitted geminiviruses. This apparent inconsistency between the recorded production in the FaoStat database and the observations reported by virologists in the field may be due to increased awareness of the damage caused by pathogens in recent years and/or the lack of recognition of their importance in the earlier years. The replanting of failed tomato crops with new plants later in the season may also contribute to the apparent discrepancy. Whether the relative importance of viral pathogens on the overall tomato production has been overstated or not, it is clear that these pathogens have the capacity to inflict heavy crop loss under conditions where epidemics of their insect vector occurs. It has been estimated that the chemical control of insects worldwide costs US$ 3-5 billion annually (James *et al.,* 1990). Despite this expenditure, crop loss due to pests and diseases has been estimated in the order of 12-13% annually.

The development of tomato varieties resistant to pests and pathogens including the whitefly-transmitted geminiviruses is one of the long-term goals of breeding programs. Wild relatives of the modern tomato including *L. peruvianum, L. hirsutum, L. pimpinellifolium,* and *L. chilense* species have been screened for the presence of naturally occurring genes for resistance to whitefly-transmitted begomoviruses. A single dominant resistance gene (*ty1c*) in *L. pimpinellifolium* (Kasrawi, 1989), a recessive tolerance gene (*ty-20*) from *L. peruvianum* (Pilowsky *et al.*, 1989), and a tolerance gene (*ty-1*) from *L. chilense* have been identified (Michelson *et al.*, 1994;

Zamir *et al.*, 1994). The introduction of these genes into commercially acceptable cultivars has been pursued during the last two decades but progress has been slow. Several alternative strategies aimed towards the engineering of pathogen-derived resistance to viruses into plants have been proposed (Lomonossoff, 1995).

Numerous studies have shown that *A. tumefaciens* Ti plasmid vectors can be used to introduce functional plant viral DNA into the genome of plants. The transformed plants carrying specific viral gene(s) provide the means to dissect any viral genome and assign function to the gene products. These studies have contributed to our understanding of the mechanisms used by viruses for replication and some of the processes leading to the expression of disease in plants.

Studies on the Australian isolate of ToLCV (Dry *et al.*, 1993; Mullineaux *et al.*, 1993; Rigden *et al.*, 1993; Rigden *et al.*, 1994; Behjatnia *et al.*, 1996; Rigden *et al.*, 1996; Dry *et al.*, 1997; Behjatnia *et al.*, 1998; Krake *et al.*, 1998; Dry *et al.*, 2000), have contributed to the molecular biology of monopartite begomoviruses, complementing studies on similar viruses in other parts of the world. An extension of this work included this current study to characterise transformed tobacco and tomato plants expressing the *c4* gene from ToLCV. Comparison of these transformed plants with similarly transformed plants with a frame-shift version of the *c4* gene construction provided direct evidence of the role of the C4 translation product in the development of viral disease symptoms associated with infection of plants by ToLCV. This work complemented the findings presented by Rigden *et al.,* 1994 on the predicted function of the C4 ORF from the monopartite begomovirus ToLCV and supports the conclusions reported in earlier studies of the C4 ORF from the curtovirus, BCTV

(Stanley and Latham, 1992; Stanley *et al.*, 1992; Latham *et al.*, 1997). Additional studies are required to understand how the C4 protein interacts with the host cellular machinery and the mechanisms operating during viral infection for temporal regulation of its expression and function. Also, similar studies are needed on other begomoviruses. For instance, it remains to be demonstrated if the ectopic expression of the *c4* gene of TYLCSV also behaves similarly to the fore-mentioned monopartite viruses. Likewise, the ectopic expression of the putative analogous *ac4* gene of bipartite begomoviruses also remains to be determined. The latter studies may influence the taxonomy of begomoviruses in the future as some studies have shown the AC4 ORF to be non-functional in the host plants tested. In some of these viruses at least, the ectopic expression of the *bc1* gene has been shown to induce virus-like symptoms in transformed plants that resemble those displayed by the *c4* phenotypes. Little is known about the function of *c4* gene, other than its proposed role in cell division.

Several of the transformed tobacco and tomato plants derived from 35*S-c4* and 35*S c4 f-s* cultures exhibited the ability to resist and tolerate ToLCV-DNA infection via agro-infection at a level of inoculum that induced disease in the similarly treated untransformed Samsun and Ailsa Craig tomato plants. These traits were unrelated to the level of expression from the *c4* or *c4 f-s* transgenes or the number of gene inserts. In fact it seemed to be unrelated to the integration of the complete transgene, as indicated by the testing by PCR. Although a Southern-blot analysis of these plants was not done, it is possible that these lines may contain fragments of viral sequence. The ability of the transformed plants to resist and tolerate the ToLCV infection may be due to insertion of the foreign sequence into a critical segment of the host genome leading to host-

mediated defence against identical sequences. In related unpublished studies, all of the kanamycin-selected T_1 generation seedlings from six independent transformed lines of 35*S*-pBin19 displayed susceptibility to agro-infection by ToLCV-DNA at the lower level of inoculum used to infect Samsun plants. This suggests that a homology-dependent sequence is a prerequisite for induced resistance and tolerance characteristics in the *c4* and *c4 f-s* transformed plants. However, it seems that this mechanism can be overcome, as indicated by the demonstrated dose-related effect of increasing the concentration of inoculum.

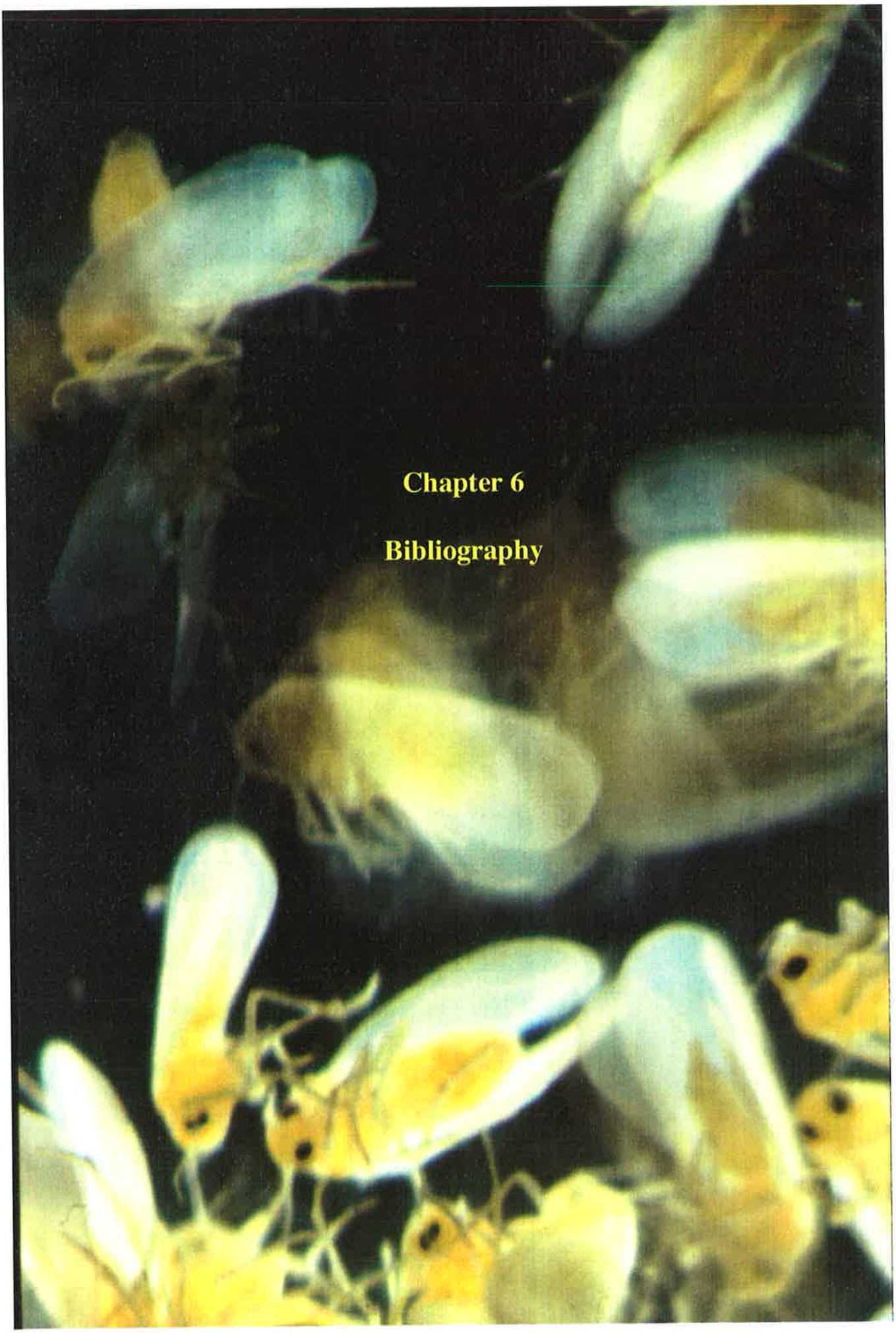
To date, there are no published studies on the potential of transgenic plants expressing the *c4* gene of either curtoviruses or begomoviruses to resist infection by viruses although several studies have demonstrated effects due to the expression of either full-length or truncated versions of the *rep* gene. At the DNA level, insertion of the *c4* gene may be viewed as a truncated *rep* sequence insert. Conserved blocks of the C4 protein sequence of unrelated geminiviruses appear to be influenced by conserved blocks of the *rep* amino acid sequence. However, the *c4* RNA transcripts are out of frame with the *rep* transcript and translation products are unique. The expression of truncated or full-length sense or antisense versions of the *rep* gene in transgenic plants have produced plants that appear normal. Some of these plants displayed tolerance or resistance to virus infection when challenged with viruliferous whiteflies or following agro-infection with infectious viral DNA (Day *et al.*, 1991; Bejarano and Lichtenstein, 1994; Hong and Stanley, 1996; Noris *et al.*, 1996; Bendahmane and Gronenborn, 1997; Brunetti *et al.*, 1997; Aragao *et al.*, 1998; Hanson and Maxwell, 1999; Sangare *et al.*, 1999). The regeneration of transgenic plants expressing a functional full-length *rep* gene in the sense orientation has often proved to be difficult (Day *et al.*, 1991; Bendahmane and

Gronenborn, 1997; Dry, Krake and Rezaian, unpublished data). Also, an attempt to express the complete set of complementary-sense genes of ToLCV in transformed Samsun tobacco yielded only two plants that expressed a transcript equivalent in size to that predicted to span the C1-C3 ORFs (Krake, Dry and Rezaian, unpublished data). These plants were not immune to infection by ToLCV-DNA via agro-infection and were not tested against whitefly-mediated virus infection.

Under natural conditions, the spread of begomoviruses is essentially via whitefly-mediated infection. As whiteflies are important pests in their own right, it seems wise to aim for effective control of the pest and as a result the viruses transmitted by them should also be contained. Some of the wild relatives of *Lycopersicon esculentum* including accessions of *L. hirsutum* and *L. pennellii* have high density of leaf trichomes or secretory trichomes that were either unattractive to whiteflies or the exudate from glandular trichomes entrapped the whiteflies before they could transmit virus (Nakhla and Maxwell, 1998). These traits introduced into commercial varieties may provide an opportunity in the longer term for the control of whiteflies.

In Israel, epidemics of whitefly-transmitted viruses have been largely avoided in the Arava region with a crop-free period of 1 month during each year (Ucko *et al.*, 1998). In the event of similar management strategy failing, the application of pathogen-derived resistance procedures may serve a useful purpose. Transformed tomato plants like those described in this study can be produced quickly and easily vegetatively propagated. Lines with the ability to resist infection or at least slow down replication of viral DNA would be useful in breaking epidemics of virus infection and permitting the development of a tomato crop.

Concerns have been raised about the safety issues associated with the release and use of genetically engineered plants. The predicted benefits and the potentially detrimental effects arising from the use of pathogen-mediated resistance mechanisms to control virus diseases have been the subject of considerable discussion in recent times. A number of diverse opinions have been expressed. Some have argued that the use of transgenes for the induction of coat protein mediated resistance in field crops presents unacceptable risks (de Zoeten, 1991), while other believe that the potential risks are negligible and manageable (Falk and Bruening, 1994). A general consensus view emerging among scientists is that while some risks are evident, the side-effects merit detailed analyses to determine probability and potential impact (Rubio *et al.,* 1999). In the final analysis, the decision on whether to deploy genetically engineered resistance should be based on the perceived agronomic and environmental risks versus the benefits of increased crop production.



Chapter 6

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