Homology-dependent gene silencing associated with infection by *Tomato leaf curl virus-Australia* (*Begomovirus: Geminiviridae*)

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Thesis submitted for the degree of Doctor of Philosophy at the University of Adelaide

October, 2003

To my family

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"Homology-dependent gene silencing associated with infection by *Tomato leaf curl virus-Australia* (*Begomovirus: Geminiviridae*)"

ABSTRACT

Tomato leaf curl virus-Australia (TLCV) promoters drive both constitutive and tissue-specific expression in tobacco. This study describes the silencing of tobacco transgenes carrying TLCV promoters following TLCV infection.

In a previous study to investigate TLCV promoter activity *in planta*, four complementary-sense (C1:GUS, C2:GUS, C3:GUS and C4:GUS) and two virion-sense (V1:GUSΔC and V2:GUSΔC) TLCV promoter:GUS transgenes were stably transformed into tobacco. Following systemic infection of the TLCV promoter:GUS plants with TLCV, transgene expression driven by all six TLCV promoters was silenced. Transgene silencing occurred in the vascular, mesophyll and floral tissues of V2:GUSΔC plants. Transgene silencing occurred with the continued replication of TLCV and was restricted to plants carrying TLCV-derived sequences, however infection of V2:GUSΔC plants by heterologous geminiviruses did not result in silencing. Thus, transgene silencing following TLCV infection was sequence-specific, requiring sequence homology between both the virus and the transgene.

Nuclear run-on assays to detect transcription from the V2:GUSΔC transgene in silenced plants indicated that silencing occurred at the level of transcription. The level of cytosine methylation of the V2:GUSΔC transgene in silenced tissue was assessed by bisulfite modification and sequencing. Following silencing, hypermethylation of cytosines in the TLCV-derived sequences of the transgene was observed. In contrast, hypomethylation of cytosines in the GUS sequences of the transgenes occurred in silenced tissue. The sequence-specific hypermethylation and transcriptional silencing of the V2:GUSΔC transgene

following TLCV infection represents the first case of virus-induced transcriptional gene silencing (VITGS) associated with a geminivirus infection.

Transgene expression was analysed in the virus-free progeny of silenced and non-silenced TLCV promoter:GUS plants. The silenced phenotype of infected V2:GUSΔC plants was inherited in progeny seedlings, however spontaneous partial restoration of transgene activity was observed with further growth. The heritable, yet reversible nature of TLCV-mediated VITGS was therefore a type of epimutation. The silenced phenotype was also inherited in V1:GUSΔC progeny from an infected parent. However, the silenced phenotype of the complementary-sense promoter:GUS plants was either partially (C1:GUS and C4:GUS) or completely (C3:GUS) reset in progeny. Interference with inherited cytosine methylation patterns and chromatin structures in C1:GUS and V2:GUSΔC progeny from infected parents indicated a role for both cytosine methylation and non-hypoacetylated heterochromatin formation in the inheritance of VITGS.

A component of the conserved antiviral RNA silencing pathway, short interfering RNAs (siRNAs), are reported to direct sequence-specific cytosine methylation in plants. siRNAs specific to transcribed TLCV sequences were detected during TLCV infection of four solanaceous host species, leading to the conclusion that TLCV infection induces the RNA silencing pathway. siRNAs homologous to the TLCV-derived V2:GUSΔC transgene sequences which became hypermethylated following VITGS were detected in tobacco following TLCV infection. Thus, TLCV-specific siRNAs were a candidate for the mechanism directing sequence-specific methylation of the TLCV promoter:GUS transgenes. siRNAs homologous to untranslated TLCV intergenic region sequences were detected in non-transgenic tobacco. This result may suggest previously uncharacterised transcription from the TLCV genome and/or the involvement of host RNA-directed RNA polymerases during the induction of RNA silencing by TLCV infection.

PUBLICATION

Seemanpillai, M.J., Dry, I.B., Randles, J.W. and Rezaian, M.A. (2003) Transcriptional Silencing of Geminiviral Promoter-Driven Transgenes Following Homologous Virus Infection. *Molecular Plant-Microbe Interactions* 16(5): 429-438

STATEMENT

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Mark Seemanpillai

October 2003

ACKNOWLEDGMENTS

I wish to thank my supervisors, Dr M. Ali Rezaian and Professor John W. Randles for their guidance and support during the course of this study. In addition to their excellent supervision, they have often gone beyond the call of duty in their assistance and encouragement.

I would also like to thank Dr Ian Dry, Mr Jamus Stonor, Dr Xue-shu Bian and all members of the Rezaian laboratory for their assistance and collaboration. Technical assistance was also kindly provided by Drs Varsha Wesley, Jean Finnegan and Peter Waterhouse of CSIRO Plant Industry.

I would like to thank my many friends at CSIRO Horticulture, especially Alan Little, Luke Selth, Dale Mayer, Barbara Alberter, Karina Swann, Diane Webster and Matthew Hayes from the lab, and Chris Davies, Susan Wheeler, Chi Hua, Matthew Tucker, Melissa Pickering, Elizabeth Lee and Mandy Walker from CSIRO Horticulture.

Excellent staff support has been provided by Mrs Maria Piscioneri, Mrs Julie Powell and Mr Tim Abbott. My gratitude also goes to Professor Anna Koltunow for her professional guidance.

I am grateful to my friends and the Prowse, Campbell and Boxhall families for their friendship and support over the years. Finally, I gratefully thank my family for their constant support and love.

ABBREVIATIONS

ACMV	*African cassava mosaic virus*
AYVV	*Ageratum yellow vein virus*
ATP	Adenosine triphosphate
AzaC	5-azacytidine
BGMV	*Bean golden mosaic virus*
bp	Base pairs
BSA	Bovine serum albumin
C1:GUS	TLCV C1 ORF promoter:GUS fusion construct
C2:GUS	TLCV C2 ORF promoter:GUS fusion construct
C3:GUS	TLCV C3 ORF promoter:GUS fusion construct
C4:GUS	TLCV C4 ORF promoter:GUS fusion construct
CaMV	*Cauliflower mosaic virus*
CLCuMV	*Cotton leaf curl Multan virus*
CP	Capsid/coat protein
CTP	Cytosine triphosphate
dH_2O	Distilled water
dpi	Days post inoculation
dpg	Days post germination
DTT	Dithiothreitol
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytosine triphosphate
dGTP	Deoxyguanosine triphosphate

dsDNA	Double-stranded deoxyribonucleic acid
dsRNA	Double-stranded ribonucleic acid
dTTP	Deoxythymidine triphosphate
EDTA	Ethylenediamine *tetra* acetic acid (disodium salt)
GFP	Green fluorescent protein
GTP	Guanosine triphosphate
GUS	β-glucuronidase
HDAC	Histone deacetylase inhibitor
HdGS	Homology-dependent gene silencing
IR	Intergenic region
IPTG	*Iso*-propyl-β-D-thiogalactopyranoside
kb	Kilobase
M	Molar
min	Minute
MSV	*Maize streak virus*
NOS	Nopaline synthase
NTP	Nucleoside triphosphate
nt	Nucleotide
ORF	Open reading frame
PCR	Polymerase chain reaction
Pd	Plasmodesmata
PHYVV	*Pepper huasteco yellow vein virus*
PVX	*Potato virus X*
RdRP	RNA-directed RNA polymerase
Rep	Replication–associated protein

RF	Replicative form
RT-PCR	Reverse transcription polymerase chain reaction
s	Second
SB	Sodium butyrate
SDS	Sodium dodecyl sulfate
siRNA	Short interfering ribonucleic acid
ssDNA	Single–stranded deoxyribonucleic acid
TEMED	N, N, N'-N'-Tetramethylethylenediamine
TGMV	*Tomato golden mosaic virus*
TLCV	*Tomato leaf curl virus-Australia*
TPCTV	*Tomato pseudo-curly top virus*
TRV	*Tobacco rattle virus*
TSA	Trichostatin A
TSS	Transcriptional start site
TTP	Thymidine triphosphate
TYLCSV	*Tomato yellow leaf curl Sardinia virus*
TYLCV	*Tomato yellow leaf curl virus*
V1:GUS	TLCV V1 ORF promoter:GUS fusion construct
V1:GUSΔC	TLCV V1 ORF promoter:GUS fusion construct (complementary-sense TLCV ORFs deleted)
V2:GUS	TLCV V2 ORF promoter:GUS fusion construct
V2:GUSΔC	TLCV V2 ORF promoter:GUS fusion construct (complementary-sense TLCV ORFs deleted)
VITGS	Virus-induced transcriptional gene silencing
X-gal	5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside

Chapter 1

General Introduction

1.1 Introduction

Geminiviruses are pathogens of many sub-tropical and tropical plants, including a number of important crop species. Accordingly, they are of considerable economic interest and the subject of efforts to control their spread through an understanding of geminivirus biology. The replication of the geminivirus DNA genome is highly reliant on host factors. Therefore, geminiviruses also provide an ideal model for the study of DNA replication in plants. A number of excellent reviews on geminivirus replication and host interaction are available (Gutierrez 2000; Gutierrez 2000a; Hanley-Bowdoin et al. 1999).

The expression of transgenes in many eukaryotic organisms is restricted by the phenomenon of homology–dependent gene silencing (HdGS). HdGS was first identified during the attempted over-expression of a pigmentation gene in the petunia plant (van der Krol et al. 1990; Jorgenesen et al. 1996). Subsequently, the phenomenon of HdGS (also known as co-suppression, post-transcriptional gene silencing, quelling, RNA interference (RNAi) and RNA silencing) has been observed for fungi, nematodes, Drosophila, zebra fish, and mammals including human cells (Bernstein et al. 2001). HdGS is also recognised as the basis for the silencing of plant transgenes following infection by viruses that share sequence homology with the affected transgene.

In this chapter an overview is provided of geminivirus classification, genome organisation and replication, with emphasis on the *Tomato leaf curl virus-Australia* (ToLCV-Au. For the purposes of this thesis, *Tomato leaf curl virus-Australia* is abbreviated to TLCV). Additionally, studies describing the silencing of plant promoter sequences following infection by viruses carrying homologous promoter sequences are discussed.

1.2 Taxonomy of the Geminiviridae

Species in the family *Geminiviridae* have a circular DNA genome and are transmitted between plant hosts by insects. They are classified into four genera. Currently, the classification of geminiviruses into genera is based upon the number of genome components (1 or 2) and the species of insect vector which transmits the virus between hosts. The genus *Mastrevirus* (type species *Maize streak virus*, MSV) includes geminiviruses with a monopartite genome which are transmitted predominantly between monocotyledonous hosts by a leafhopper insect vector. The genus *Curtovirus* (type species *Beet curly top virus*, BCTV) includes geminiviruses with a monopartite genome which are transmitted between dicotyledonous hosts by a leafhopper vector. The genus *Topocuvirus* has one member, *Tomato pseudo-curly top virus* (TPCTV), which is transmitted by a treehopper vector. The largest genus *Begomovirus* (type species *Bean golden mosaic virus*, BGMV) includes geminiviruses which have either a monopartite or bipartite genome and are transmitted between dicotyledonous hosts by the whitefly *Bemisia tabaci* (Gennadius).

1.3 Particle morphology and composition

The geminivirus virion is characterised by a twinned "geminate" structure, which consists of two joined, incomplete icosahedra (van Regenmortel et al. 2000). Each virion is presumed to encapsidate a single DNA molecule of 2.5-3.0 kb. Thus, for bipartite geminiviruses presumably two virions (each encapsidating a separate genome component) are required for infection. Each virion contains a single structural protein, the virus capsid protein. The first determination of the structure of a geminivirus virion (MSV) has confirmed the "geminate" particle model and suggests that 110 copies of the capsid protein are required for construction of each virion particle (Zhang et al. 2001).

1.4 Genome organisation of the geminiviruses

1.4.1 General organisation of the geminivirus genome

Replication of the geminivirus genome occurs by the conversion of the virus circular ssDNA to a circular double-stranded (ds) DNA replicative form (RF). The virus ORFs are encoded on both the virion-sense and complementary-sense strands of the RF. The small size of the geminivirus genome dictates the efficient organisation of the virus genes. Consequently, virus ORFs are often overlapping. In all geminiviruses, a small (approximately 300 bp) intergenic region (IR) is found between the 5'-ends of the virion- and complementary-sense ORFs. The IR contains the virus origin of replication and is also a bi-directional promoter for the adjacent ORFs.

1.4.2 *Mastrevirus*

The 2.6-2.8 kb monopartite genome of the mastreviruses encodes four ORFs, which direct the synthesis of three (possibly four) virus proteins (van Regenmortel et al. 2000). In contrast to other geminivirus genera, mastrevirus genomes encode a second smaller IR in addition to the IR containing the virus origin of replication.

1.4.3 *Curtovirus*

The 2.9-3.0 kb monopartite genome of curtoviruses encodes seven ORFs, which direct the synthesis of 6-7 virus proteins (van Regenmortel et al. 2000). The curtovirus genome encodes one IR.

1.4.4 *Topocuvirus*

The 2.8 kb genome of the only member of the topocuvirus genus, TPCTV, encodes six ORFs (Briddon et al. 1996). It is not known how many virus proteins are produced during TPCTV infection. The TPCTV genome encodes one IR.

1.4.5 *Begomovirus*

Begomoviruses are unique amongst geminiviruses in having a large number of species with two genome components, DNA A and DNA B. Both DNAs are between 2.5-2.8 kb (van Regenmortel et al. 2000). The begomovirus DNA A is similar in the organisation of ORFs to the monopartite genome of curtoviruses and encodes 5-6 ORFs. The DNA B of bipartite begomoviruses encodes two ORFs, one on either strand of the DNA B RF. The DNA A and B genome components share approximately 200 bp of sequence from the IR of DNA A, including the virus origin of replication. The 2.5-2.8 kb genome of the monopartite begomoviruses is almost identical in the organisation of virus ORFs to the DNA A component of the bipartite begomoviruses.

1.4.5.1 Begomovirus satellite DNA: the geminivirus disease complex

A minority of begomoviruses have a monopartite genome. Inoculation with the cloned component of the monopartite *Tomato yellow leaf curl virus* (TYLCV) causes typical disease symptoms and generates viable virus progeny, thereby fulfilling Koch's postulates (Navot et al. 1991). However, inoculation with the cloned components of the monopartite begomoviruses *Ageratum yellow vein virus* (AYVV) and *Cotton leaf curl Multan virus* (CLCuMV) does not result in typical disease symptoms and so does not fulfil Koch's postulates.

A novel circular ssDNA component was isolated from plants during TLCV infection and identified as the first satellite associated with a DNA virus (Dry et al. 1997). The TLCV

satellite was approximately one-quarter of the size of the TLCV genome, contained sequences resembling the virus origin of replication, did not contain extended ORFs and was not required for typical disease symptom development. The satellite DNA was reliant on the TLCV helper component for replication and encapsidation.

A second begomovirus satellite DNA was associated with infection by CLCuMV. The circular ssDNA component, named DNA 1, was one-half of the size of the helper component and contained the CLCuMV C1 ORF, allowing the DNA 1 to replicate autonomously (Mansoor et al. 1999). Encapsidation of the DNA 1 was reliant on the helper virus. Subsequently, a DNA 1 component was associated with infection by AYVV (Saunders and Stanley 1999). However, co-infection with CLCuMV or AYVV and the associated DNA 1 did not result in typical disease symptoms. Thus the aetiology of either disease was not resolved by the detection of a DNA 1 component.

Further analysis of circular ssDNA components associated with AYVV infection identified another component, subsequently named DNA β. AYVV DNA β was one-half the size of the helper component and contained no homology to the helper virus apart from the origin of replication (Saunders et al. 2000). Subsequently, a DNA β was associated with infection by CLCuMV (Briddon et al. 2001). Co-infection of AYVV or CLCuMV with the associated DNA β resulted in the typical disease symptoms for each virus, thus resolving the aetiology of both diseases.

The identification of geminivirus disease complexes comprising monopartite begomoviruses and DNA β components may be of importance to the epidemiology of diseases caused by monopartite begomoviruses. The *Sri Lankan cassava mosaic virus* (SLCMV) is normally limited to infection of cassava and does not replicate in ageratum (*Ageratum conyzoides*). However co-infection of SLCMV with AYVV DNA β in ageratum allowed the replication of SLCMV and development of disease symptoms (Saunders et al. 2002). Thus AYVV DNA β

can support the replication and spread of heterologous begomoviruses, thereby facilitating an increase in host range of some geminiviruses. A excellent review on the implications of the geminivirus-DNA β complex is available (Mansoor et al. 2003).

1.5 Significance of geminiviruses

The begomoviruses are the most important disease-causing group amongst geminiviruses. The whitefly-transmitted begomoviruses cause significant, often total yield losses of important food and industrial crops in tropical and sub-tropical agro-ecosystems around the world (Morales and Anderson 2001). Although begomoviruses have been recognised as a constraint to agricultural production for many decades, the last twenty years have seen a re-emergence of begomoviruses as plant pathogens. Both new and benign begomoviruses (those species that were previously believed to have limited potential for the invasion of crop species) have been responsible for new disease epidemics in not only sub-tropical but also more temperate agro-ecosystems (Brown 2000).

Suggestions to explain the new geminivirus epidemics (most notably seen in Uganda and adjacent African nations) include the generation of new species by either recombination or pseudo-recombination between geminiviruses in mixed infections (Zhou et al. 1997; Schnippenkoetter et al. 2001). Also, variants of the whitefly vector of begomoviruses, *Bemisia tabaci*, have been observed to differ in their capacity to transmit certain begomoviruses. This is exemplified by the spread of the exotic B biotype of *B. tabaci*. The B biotype likely originated in the Eastern hemisphere and has spread to the Western hemisphere, probably by the propagation of ornamental plants originating from Israel (Brown 2000). The movement of the B biotype out of the Eastern hemisphere during the early 1990's has coincided with the emergence for the first time of tomato- and pepper-infecting begomoviruses in the America's. The B biotype is characterised by a greater fecundity and

host feeding range than previous *Bemisia* biotypes (de Barro and Andersen 1996) and this has probably driven the emergence of new diseases caused by whitefly-transmitted geminiviruses. Thus, the spread of a highly efficient insect vector and the ability of begomoviruses to rapidly adapt to new plant hosts has combined to make the whitefly-transmitted geminiviruses into the "pest of the century" (Morales and Anderson 2001).

1.6 Geminiviruses causing leaf curl disease in tomato

1.6.1 Geographical distribution

The most recent revision of species within the genus *Begomovirus* lists eleven geminiviruses as causing leaf curl disease in tomato and are grouped with the Old World begomoviruses (Fauquet et al. 2003). Geminiviruses causing tomato leaf curl (TLC) disease are found in India, Sri Lanka, Bangladesh, Malaysia, Laos, Vietnam, Malaysia and Australia and include both monopartite and bipartite viruses. Other viruses tentatively assigned as TLCV species have been isolated from Indonesia, Nicaragua, the Philippines, Senegal, Mexico and Tanzania (Fauquet et al. 2003).

The first incidence of TLC disease in Australia was recorded in 1970 at a site near Darwin, Northern Territory (Behjatnia et al. 1996). Initially, the TLC disease caused severe to complete yield losses in the local tomato industry. Transmission of the disease was by the indigenous biotype of *B. tabaci*, which colonizes large areas of Australia between the Northern Territory and north Queensland. The genomic component of the TLC disease agent was cloned and identified as a monopartite begomovirus (Dry et al. 1993). The virus was named *Tomato leaf curl virus*. Monitoring of the occurrence of TLCV has recorded the movement of the virus westward through the regions infected by indigenous *B. tabaci*, to the Lakeland district of far north Queensland (Stonor et al. 2003).

The B biotype of *B. tabaci* was recorded in Australia in 1994 and has infested large areas of the eastern Australian coastal regions (de Barro and Andersen 1996), which include the intensive tomato cropping regions of Australia. Infestation by the B biotype whitefly has reached regions of Queensland immediately south of the Lakeland district. The eventual likely overlap between TLCV-infected and B biotype whitefly-infested regions will facilitate the rapid movement of TLCV into the main tomato-growing regions of Australia, threatening the multi-million dollar tomato industry.

1.6.2 Transmission

Begomoviruses are transmitted exclusively between plant hosts by the whitefly, *Bemisia tabaci* (Gennadius) (Homoptera/Hemiptera: Aleyrodidae). Once acquired, whiteflies transmit begomoviruses in a persistent-circulative manner and the virus remains associated for the entire adult life of the insect. Recently, whiteflies have been shown to transmit the monopartite begomovirus TYLCV to progeny insects (Ghanim et al. 1998) and to other whiteflies during sexual reproduction (Ghanim and Czosnek 2000). In both cases, the newly infected insects were able to transmit TYLCV to plant hosts.

The majority of tomato-infecting begomoviruses are not mechanically transmissible. Exceptions include the *Tomato golden mosaic virus* (TGMV), *Tomato mottle virus* and *Tomato yellow mosaic virus* (Behjatnia 1997). TLCV is not seed- or soil-transmissible.

1.6.3 Host range

The host range of TLC-causing geminiviruses is generally restricted to solanaceous plant hosts. However, a number of weed species from the genera *Euphorbia*, *Acanthospermum*, *Ageratum* and *Parthenium* are reservoir hosts for the isolate of TLCV occurring in southern India (Saikia and Muniyappa 1989). A comprehensive test of plants infected by the Australian isolate of TLCV identified a number of symptomatic and asymptomatic hosts (Stonor et al.

2003). Hosts exhibiting TLC symptoms included datura (*Datura stramonium*), tomato (*Lycopersicon esculentum*), *Nicandra physalodes*, *Nicotiana tabacum*, *Petunia hybrida* and *Physalis virginiana*. Hosts that remained asymptomatic during TLCV infection included *Capsicum frutescens*, *Cyphomandra betacea*, *Phaseolus vulgaris*, *Solanum pseudocapsicum* and *Solanum seaforthianum*. Both a *L. esculentum* cultivar (cherry tomato) and *C. frutescens* (birds-eye chilli) are feral plants occurring throughout northern Australia. Symptomatic hosts of TLCV also include the experimental hosts *Nicotiana benthamiana* and *Nicotiana clevelandii*.

1.6.4 Symptoms of TLCV infection

Conde and Connelly (1994) described the symptoms of TLCV in tomato plants as follows:

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

1.6.5 TLCV and its relationship to other geminiviruses

The Australian isolate of TLCV is a monopartite begomovirus of 2766 nt. The replicative form encodes six ORFs and is depicted in Figure 1.1. The genome organisation of TLCV conforms closely to the genomic organisation of other dicotyledonous-infecting monopartite geminiviruses by having two virion-sense and four complementary-sense ORFs, and one IR containing the geminivirus origin of replication.

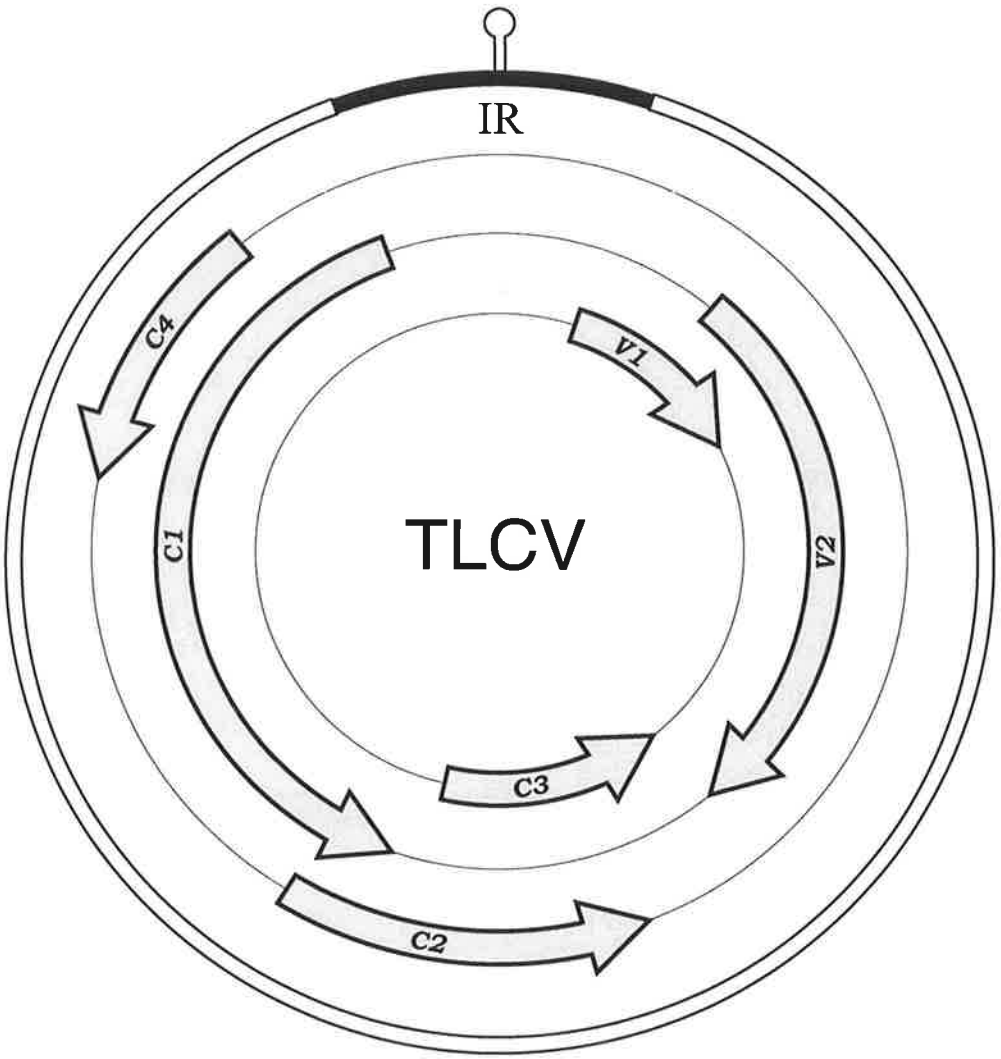

Figure 1.1 Genome organisation of TLCV. ORFs on the virion-sense (clockwise) strand and the complementary-sense (anticlockwise) strand are displayed by arrows. The positions of the conserved stem-loop structure (♀) and intergenic region (IR) are also marked.

An alignment of the conserved geminivirus motifs present in the TLCV IR against published geminivirus sequences showed TLCV to be most similar to the Indian isolate of TYLCV (75.9 % similarity) and the DNA A component of ACMV (71.9 % similarity) (Dry et al. 1993). However, the alignment of certain TLCV ORFs by amino acid identity showed TLCV to be most similar to the DNA A component of ACMV (Dry et al. 2000).

1.7 Geminivirus gene functions

Much progress has been made in determining the structure and function of geminivirus gene products. Generally, geminiviruses encode only one structural protein, the capsid protein. Other gene products are involved in the replication and/or movement of virus DNA, transcription of virus ORFs or interaction with the host environment to allow or enhance proliferation of the virus. Geminivirus gene products exhibit multifunctionality and each product can be associated with multiple stages of the virus life cycle. In this section, studies of geminivirus gene function are summarised and presented in a series of tables for each virus gene product. An excellent review of geminivirus replication and host interaction is available (Hanley-Bowdoin et al. 1999). Except where specified, the results presented in the tables below are taken from this review. For recent review of the functions and interactions of mastrevirus gene products see Boulton (2002).

1.7.1 V1 ORF

The intercellular spread of geminiviruses requires the movement of an infectious form of virus DNA to be transported from the nucleus of an infected cell to a contiguous uninfected cell via the plasmodesmata (Pd). Typically, Pd size exclusion limits would prevent transport of macromolecular complexes of the size of a virus genome between adjacent plant cells. Therefore the intercellular spread of geminiviruses is likely to require the enlargement of Pd exclusion limits, or the modification of Pd to allow the active transport of the virus genome.

This function may be provided by the V1 gene product of monopartite geminiviruses, also known as the pre-coat or movement protein. For bipartite geminiviruses, the C1 gene product of DNA B may mediate the intercellular spread of the virus and functionally replace the V1 gene product of monopartite geminiviruses. For a recent review of geminivirus movement proteins, see Gafni and Epel (2002).

Table 1.1 Summary of the putative functions of the geminivirus V1 gene product.

Virus infection cycle	*Putative function*	*Reference*
Geminivirus genome replication	• Accumulation of virus ssDNA • Determinant of symptom severity • Accumulation of virus dsDNA	(Wartig et al. 1997) (Rigden et al. 1993) (Rigden et al. 1993)
Interaction with virus proteins	• Interaction with coat protein	(Liu et al. 2001)
Interaction with host	• Localization to plasmodesmata • Stunting of plant growth	(Kotlizky et al. 2000) (Selth et al. 2003)

1.7.2 V2/AR1 ORF

The capsid or coat protein (CP) (TLCV V2 ORF gene product) of geminiviruses is the structural protein for virion particle assembly. Upon the initial entry of a geminivirus into a host cell, virus ssDNA must be targeted to the nucleus for replication of the virus genome. For monopartite geminiviruses, the CP is likely to mediate the movement of the virus ssDNA genome into and out of the host nuclei and is indispensable for systemic infection by the virus. For bipartite geminiviruses, the CP is dispensable for systemic infection and the virion-sense gene product from DNA B appears to mediate movement of the virus ssDNA through the nuclear membrane. For a recent review, see Gafni and Epel (2002).

Table 1.2 Summary of the putative functions of the geminivirus V2/AR1 gene product.

Virus infection cycle	*Putative function*	*Reference*
Geminivirus genome replication	• Accumulation of virus ssDNA • Binding to ssDNA with high affinity in sequence-independent manner	(Wartig et al. 1997) (Rigden et al. 1993) (Palanichelvam et al. 1998)
Interaction with virus proteins	• Oligomerisation	(Hallan and Gafni 2001)

Interaction with host	• Localization to nuclei of infected cells	(Kunik et al. 1998)
Interaction with insect vector	• Determinant of insect vector specificity • Interaction with GroEL protein of whitefly endosymbiont bacteria	(Morin et al. 2000) (Morin et al. 2000)
Assembly	• Encapsidation of virus ssDNA	

1.7.3 C1/AL1 ORF

The C1/AL1 gene product, Rep, plays a key role in geminivirus DNA replication and transcription. Rep is the only virus protein absolutely required for the replication of geminivirus DNA in both plant and bacterial hosts (Rigden et al. 1996) and confers virus-specific recognition of its cognate origin of replication (Hanley-Bowdoin et al. 1999). Additionally, the interaction of Rep with host factors appears to result in interference with the host cell cycle to prepare the infected cell for virus replication.

Table 1.3 Summary of the putative functions of the geminivirus C1/AL1 gene product.

Virus infection cycle	*Putative function*	*Reference*
Geminivirus genome replication	• Localisation to nuclei of infected cells • Cleavage and ligation of ssDNA plus-strand DNA in loop of hairpin motif • Low affinity binding of stem-loop motif of intergenic region • NTPase activity	(Castellano et al. 1999)
Regulation of virus gene expression	• High affinity dsDNA binding of motifs in geminivirus intergenic region • Repression of own expression at the level of transcription • Activation of virion-sense gene expression in some geminiviruses	(Castellano et al. 1999)
Interaction with virus proteins	• Oligomerisation to form complexes with approximately eight subunits • Binds to C3 protein	
Interaction with host	• Induction of expression of the DNA synthesis protein, proliferating cell nuclear antigen, in non-dividing cells • Interaction with plant	

	retinoblastoma-related protein • Interaction with NAC-like GRAB proteins • Interaction with wheat replication factor C • Interaction with Ser/Thr kinase, kinesin and histone H3 proteins • Hypersensitive response in plants	(Xie et al. 1999) (Luque et al. 2002) (Kong and Hanley-Bowdoin 2002) (Selth et al. 2003)

The mastreviruses are unique amongst geminiviruses for encoding two Rep proteins, Rep and RepA. The Rep proteins of mastreviruses are produced from a spliced complementary-sense transcript that fuses the overlapping C1 (Rep) and C2 (RepA) ORFs (Boulton 2002). The mastrevirus *Bean yellow dwarf virus* replicated more efficiently following mutation of the RepA protein, suggesting that RepA might control the level of viral replication (Liu et al. 1998).

Table 1.4 Summary of the putative functions of the mastrevirus C2 gene product.

Virus infection cycle	*Putative function*	*Reference*
Geminivirus genome replication	• Repressor of virus genome replication	(Liu et al. 1998)
Regulation of virus gene expression	• Enhancement of virion-sense gene expression	(Zhan et al. 1993) (Munoz-Martin et al. 2003)
Interaction with virus proteins	• Oligomerisation • Interaction with Rep	(Horvath et al. 1998) (Boulton 2002)
Interaction with host	• Interaction with plant retinoblastoma-related protein • Interaction with proteins containing NAC-domains • General activator of transcription	(Liu et al. 1999) (Xie et al. 1999) (Horvath et al. 1998)

1.7.4 C2/AL2 ORF

The C2/AL2 ORF gene product is a transcriptional activator protein and is abbreviated to TrAP (Hanley-Bowdoin et al. 1999). TrAP is likely to be involved in the regulation of late-gene expression in geminiviruses. Additionally, the interaction of TrAP with host factors

appears to impinge on host processes, thereby creating a cellular environment conducive for geminivirus replication.

Table 1.5 Summary of the putative functions of the geminivirus C2/AL2 gene product.

Virus infection cycle	*Putative function*	*Reference*
Geminivirus genome replication	• Localization to nuclei of infected cells • Enhancement of accumulation of virus DNA	
Regulation of virus gene expression	• Transactivation of virion-sense gene expression at the level of transcription	(Sunter and Bisaro 2003)
Interaction with host	• Suppressor of host antiviral RNA silencing response • Enhancement of host susceptibility to plant viruses • Interaction with SNF1, a global regulator of plant metabolism • Local and systemic necrosis in plants	(Voinnet et al. 1999) (Sunter et al. 2001) (Hao et al. 2003) (Selth et al. 2003)
Other	• Binding to dsDNA with low affinity in sequence-independent manner • Binding to ssDNA with high affinity in sequence-independent manner • General activation of transcription • Binding to zinc	(Hartitz et al. 1999)

1.7.5 C3/AL3 ORF

The C3/AL3 ORF gene product is associated with the enhancement of geminivirus DNA replication and is abbreviated to REn.

Table 1.6 Summary of the putative functions of the geminivirus C3/AL3 gene product.

Virus infection cycle	*Putative function*	*Reference*
Geminivirus genome replication	• Localizes to nuclei of infected cells • Enhances accumulation of virus DNA	
Interaction with virus proteins	• Oligomerisation • Interaction with Rep	
Interaction with host	• Interaction with plant retinoblastoma protein	(Settlage et al. 2001)

	• Stunting of plant growth	(Selth et al. 2003)

1.7.6 C4/AL4 ORF

The function of the C4 ORF gene product of geminiviruses is largely unknown. The growth abnormalities observed in plants ectopically expressing the C4 protein (Krake et al. 1998) may be due to disruption of the cell cycle control through the specific interaction of C4 with one (or more) cell growth regulatory pathways (Gutierrez 2000). Disruption of the TYLCV C4 ORF prevented systemic infection in tomato, indicating the involvement of this ORF in virus movement (Jupin et al. 1994).

Table 1.7 Summary of the putative functions of the geminivirus C4/AL4 gene product.

Virus infection cycle	*Putative function*	*Reference*
Interaction with host	• Abnormal cell division • Determinant of symptom severity • Localization to cell periphery • Determinant of virus movement	(Krake et al. 1998) (Selth et al. 2003) (Rojas et al. 2001) (Jupin et al. 1994)

1.8 Geminivirus DNA replication

Replication of the geminivirus genome occurs in the nucleus of infected cells. The small genome of geminiviruses does not encode a DNA polymerase, thus geminiviruses are assumed to be reliant on the host for DNA replication factors. The replication of the virus genome is initiated by Rep along with host factors, and leads to the production of new dsDNA and ssDNA virus forms. Geminivirus DNA replication has been reviewed by Hanley-Bowdoin (1999) and Gutierrez (2000; 2000a; 2002). The initial model for geminivirus replication was based on the rolling circle replication (RCR) mechanism employed by bacteriophage. Recently, a recombination-dependent replication (RDR) model for geminivirus DNA replication has been proposed (Jeske et al. 2001). A brief summary of both proposed models for geminivirus DNA replication is presented below.

1.8.1 Rolling circle replication

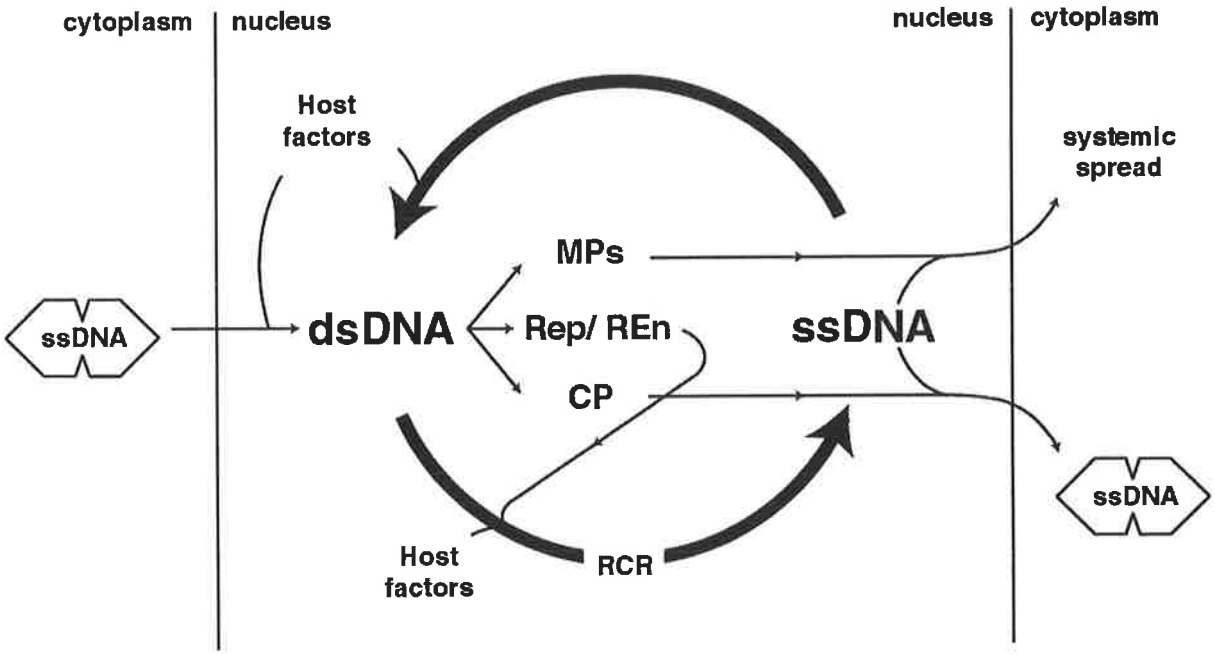

Figure 1.2. Simplified model of geminivirus DNA replication. Diagram represents invasion of a plant cell by a geminivirus virion, which is translocated from the cytoplasm to the cell nucleus. Conversion of the single-stranded DNA (ssDNA) genome to a double-stranded DNA (dsDNA) replicative form is followed by virus gene expression to synthesise the virus movement proteins (MPs), replication-associated proteins (Rep/REn) and coat protein (CP). Virus ssDNA is produced from the dsDNA replicative form by a rolling-circle replication (RCR) mechanism. The newly synthesised ssDNA can enter into one of three pathways: conversion to the dsDNA replicative form; association with the virus MPs and systemic spread via plasmodesmata to adjacent cells; or encapsidation by CP to generate new virion particles.

Figure 1.2 presents a schematic of geminivirus DNA replication as suggested by the RCR model, based on the proposed function of geminivirus proteins described above. Following entry of the virion into the plant cell, the ssDNA is imported into the nucleus. It is not known whether decapsidation of the ssDNA occurs prior to nuclear import, however it is likely that virus CP is involved in the movement into the nucleus (Gafni and Epel 2002).

The first step of RCR is presumed to be the host-directed synthesis of the complementary-sense DNA from the ssDNA template, to produce the dsDNA replicative form (RF). As virus

non-structural proteins have not been found in virions (Zhang et al. 2001), it is assumed that they are not required for complementary-sense DNA synthesis.

The geminivirus RF is the template for both the transcription of the virus ORFs (which are encoded in both strands of the RF) and the further synthesis of virus ssDNA. Virus gene expression results in the *de novo* synthesis of the replication-associated proteins (Rep and Ren), proteins associated with systemic spread of virus DNA throughout the host (for example, the proteins expressed from the DNA B of begomoviruses) and the coat protein.

The second step of RCR is initiated by Rep, which introduces a nick in the virion-sense strand of the RF specifically within the invariant nonanucleotide that is present in the stem-loop motif of all geminiviruses. The covalent linkage of the 5'-end of the nicked virion-sense strand to a tyrosine residue within Rep (Laufs et al. 1995) is likely followed by the host factor-mediated elongation from the 3'-end of the nick site, which results in the displacement of parental virion-sense ssDNA. Genome sized units of virus ssDNA are produced from the resultant concatemeric ssDNA through the site-specific nicking and ligating activities of Rep, completing the DNA replication cycle (Heyraud-Nitschke et al. 1995).

Association of the newly-synthesized virus ssDNA and coat protein may result in the assembly of new virions, thus allowing the spread of the geminivirus amongst plant hosts via the insect vector. Alternatively, the ssDNA may re-enter the virus DNA replication pathway to generate RF DNA, thus amplifying the pool of RF DNA within the infected nucleus. The systemic spread of the geminivirus throughout the host plant requires an infectious form of the virus to be exported from the nucleus and move to adjacent cells. It is not known whether the virus ss- or dsDNA is involved in cell-to-cell spread, however the requirement of virus MPs for spread suggests that a nucleoprotein complex formed of virus DNA and MPs is transported from the nucleus of an infected cell to adjacent cells via plasmodesmata (Noueiry et al. 1994; Gafni and Epel 2002).

1.8.2 Recombination-dependent replication

Recently Jeske and co-workers (2001) have proposed an additional mechanism to RCR for the replication of geminivirus DNA, which has been described as recombination-dependent replication (RDR). Replication intermediates consistent with RDR have been described for the *Abutilon mosaic virus* (AbMV), ACMV, TGMV and TYLCV (Preiss and Jeske 2003). Heterogenous AbMV DNAs migrating more slowly during fractionation than AbMV open circle DNA forms were identified by two-dimensional gel electrophoresis and electron microscopy as dimeric supercoiled AbMV dsDNA linked to heterogenous linear AbMV dsDNA, referred to as Z intermediates. The Z intermediates were observed in tissue containing actively replicating virus and did not require the AbMV AC2, AC3 ORFs or DNA B component for formation.

The RCR model for geminivirus DNA replication does not require the formation of Z intermediates. Therefore the Z intermediates were proposed to be required for RDR, in which incompletely formed virus ssDNA recombines with dimeric supercoiled dsDNA at regions of homology. The ssDNA undergoes elongation, followed by complementary-sense DNA replication mediated by host factors to produce linear dsDNA.

Geminivirus DNA replication by RDR has been suggested to explain the phenomenon of recombination frequently observed in geminiviruses (Jeske et al. 2001). Furthermore, the RDR mechanism allows the rescue of incomplete geminivirus ssDNA to generate full-length dsDNA, which is presumably not possible via the RCR mechanism.

Recent experiments in the laboratory of Dr Ali Rezaian (CSIRO Plant Industry, South Australia) have identified TLCV DNA forms that are indicative of replication by the RDR mechanism (B. Alberter, unpublished results).

1.9 Geminivirus transcription

Transcription from the geminivirus genome has been characterised for a number of viruses, including MSV (Mazithulela et al. 2000; Nikovics et al. 2001; Munoz-Martin et al. 2003), BCTV (Frischmuth et al. 1993), AbMV (Frischmuth et al. 1991) and TGMV (Hartitz et al. 1999; Sunter and Bisaro 2003). In this section, a summary of the analysis of transcription from the TLCV genome and the activity of TLCV promoters in host tissue will be presented.

1.9.1 TLCV transcription

The monopartite genome of TLCV is bi-directionally transcribed, with polycistronic virus transcripts initiating downstream of consensus TATA box sequences. Two virion- and two complementary-sense transcripts are produced from the TLCV genome during infection in tomato. These have been characterized using a combination of nuclease protection assays and rapid amplification of complementary ends (RACE)-PCR. Figure 1.3a reproduces the map of virus transcripts in relation to the TLCV genome as depicted by Mullineaux et al. (1993).

The TLCV V1, C1 and C2 gene products could be produced from the identified transcripts using the scanning model of translation. However, alternative mechanisms of translation (possibly including frame-shifting or initiation from an internal translation sequence) are likely to be employed for the translation of the V2, C3 and C4 gene products (Mullineaux et al. 1993).

The mapped 3'-ends of the virion- and complementary-sense transcripts indicated that they overlapped. This suggested that complex spatial and temporal regulation of transcription from the TLCV genome was required for proper expression of the virus gene products. A lack of agreement was found between the nuclease protection assay and RACE-PCR results for the 5'-ends of the longer complementary-sense transcript. Figure 1.3b depicts the two alternative

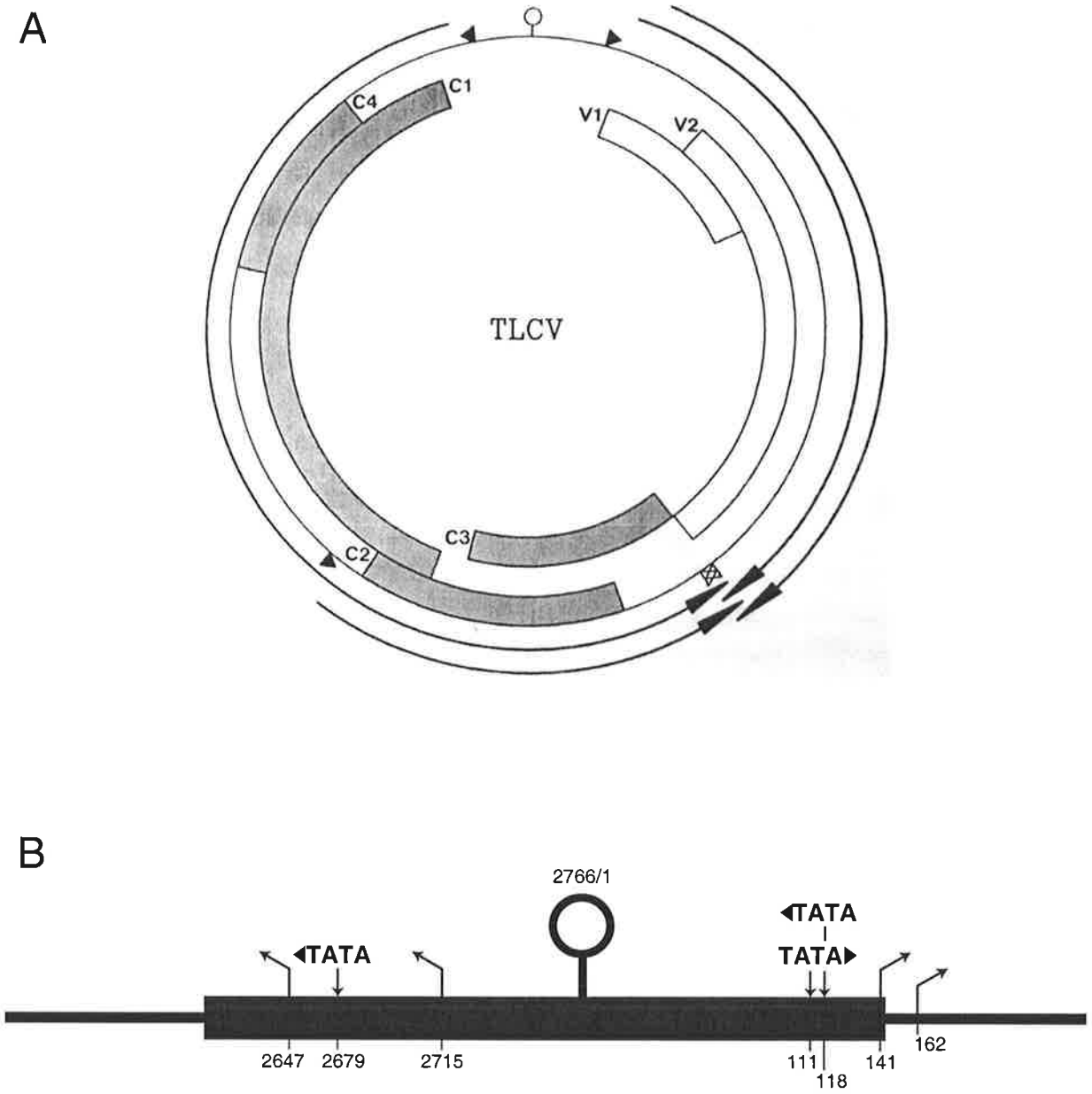

Figure 1.3 Transcription of the TLCV ORFs. All co-ordinates are from Dry et al. (1993) (A), The major virus-specific transcripts mapped onto the TLCV genome. The co-ordinates at which the ORFs begin and end are as follows: V1, 148-492; V2, 308-1075; C1, 2615-1530; C2, 1627-1223; C3, 1479-1078; C4, 2464-2159. The putative TATA sequences (▶) begin at co-ordinates 2678 (for C1), 1684 (for C2) and 111 (for V1). The putative polyadenylation sequences (▷) begin at co-ordinates 1077 and 1096 for virion- and complementary-sense transcripts respectively. The head of the conserved, potential stem loop structure (♀) begins at co-ordinate 1 (reproduced from Mullineaux et al. 1993). (B), Schematic presentation of transcriptional start sites (TSS) associated with the TLCV intergenic region. Linearised, double-stranded, replicative form of part of the genome is shown by a thin line. The intergenic region (IR) containing the origin of replication, including the conserved geminiviral stem-loop motif, is shown as a thick line. TSS mapped to the IR are shown by curved arrows. The first nucleotide of conserved TATA box sequences are marked. The likely direction of transcription directed by each TATA box sequence is shown by arrows (▶).

transcriptional start sites (TSS) (at TLCV co-ordinates 2647 and 2715) identified for the longer complementary-sense transcript, which are positioned on either side of the putative complementary-sense TATA box sequence (beginning at co-ordinate 2679). Thus it is possible that TSSs for the complementary-sense transcripts exist other than those shown in Figure 1.3a (Mullineaux et al. 1993).

1.9.2 TLCV gene expression in host tissue

A comprehensive study of TLCV promoter function and regulation was done recently by Dry and co–workers (2000). Virion– and complementary–sense TLCV promoters, linked to the β-glucuronidase (GUS) reporter gene, were stably transformed into tobacco (*Nicotiana* tabacum) and the activity and spatial expression pattern of each promoter was examined.

1.9.2.1 Construction of TLCV promoter:GUS fusion constructs

Essentially three sets of TLCV promoter:GUS fusions were constructed: complementary–sense promoter:GUS; virion–sense promoter:GUS and truncated virion–sense promoter:GUS (Figure 1.4). Complementary–sense fusions (C1:GUS, C2:GUS, C3:GUS and C4:GUS) were essentially monomeric, full-length TLCV DNA, deleted from the 3' end of the IR to mid-V2 ORF, with GUS inserted as an in–frame, N–terminal translational fusion with each complementary–sense ORF. Virion–sense fusions (V1:GUS and V2:GUS) were monomeric, full-length TLCV DNA with GUS inserted into each virion–sense ORF as described above. The truncated virion–sense fusions (V1:GUSΔC and V2:GUSΔC) were identical to the respective virion–sense fusions except for a deletion from mid-V2 ORF to mid-C1 ORF, which removed the C2 and C3 ORFs. It is important to note that upon generation of transgenic tobacco tissue, difficulty in selecting GUS–positive specimens was encountered for the C2:GUS (<9 % of transformants), V1:GUS (<10 % of transformants) and V2:GUS constructs (no transformants).

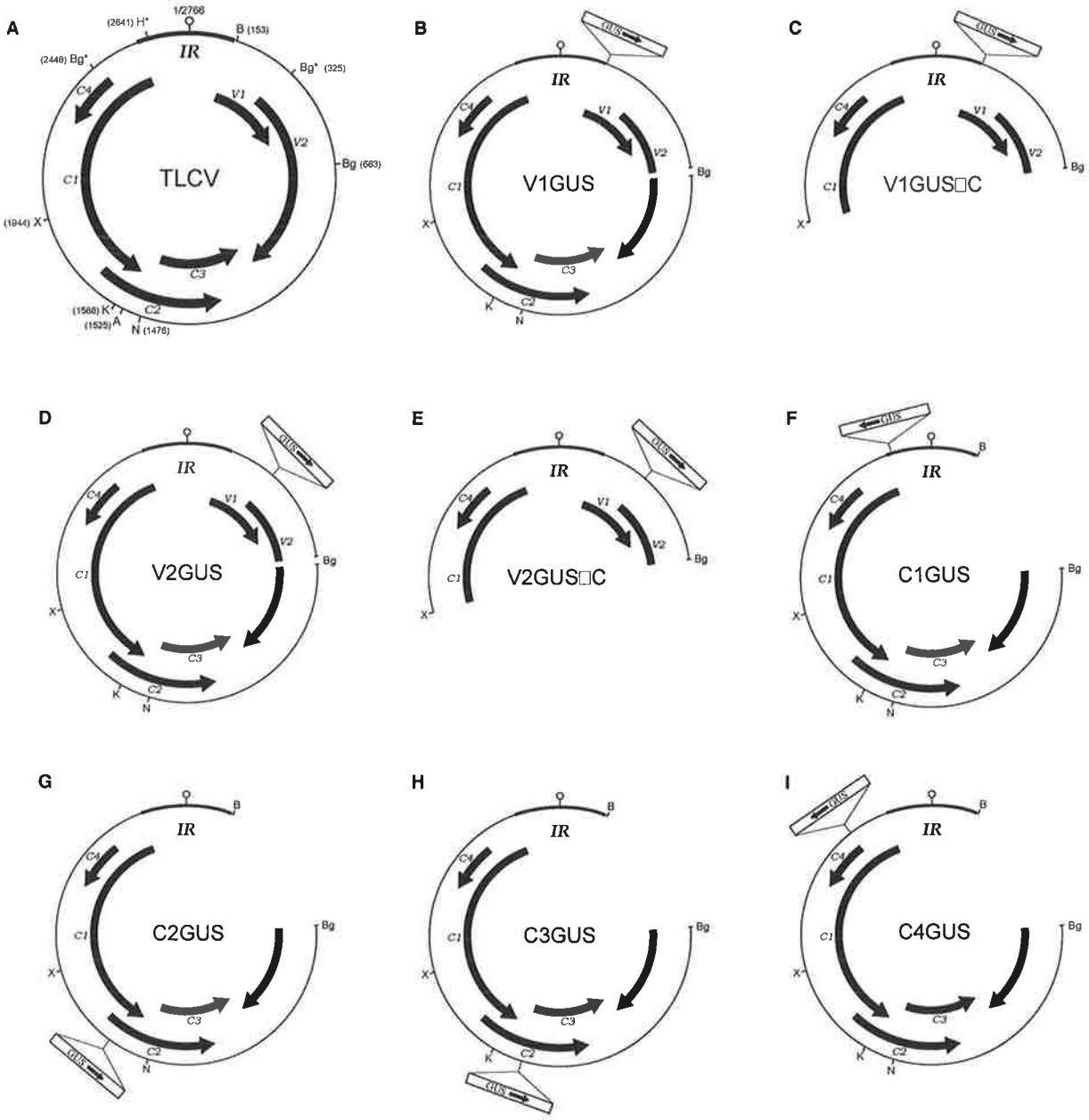

Figure 1.4 Schematic representation of TLCV promoter:GUS fusion constructs. (A), TLCV genome organization. Linearised, double-stranded, replicative form of the genome is represented by a thin line. The intergenic region (IR) containing the origin of replication, including the conserved geminiviral stem-loop motif, is shown as a thick line. Arrows denote the four complementary-sense (C1, C2, C3 and C4) and two virion-sense (V1, V2) viral open reading frames. Position of restriction sites used for construction of the TLCV:GUS fusions are indicated: B, *BamH I*; Bg, *Bgl II*; N, *Nco I*; A, *Asn I*; K, *Kpn I*; X, *Xba I*; H, *Hind III*. An asterisk (*) indicates restriction sites introduced by site-specific mutagenesis. (B-I), Promoter:GUS fusion constructs cloned into pBIN19 for tobacco transformation. The open box (GUS→) represents a 2.1-kb fragment containing a GUS-NOS terminator cassette (reproduced from Dry et al. 2000).

1.9.2.2 The TLCV promoter:GUS fusions direct GUS expression in tobacco plants

The mean activity of TLCV promoter:GUS fusions *in planta* relative to the most active transgene (V2:GUSΔC) were as follows: CaMV 35S:GUS (220 %[1]); V2:GUSΔC (100 %); C4:GUS (88 %); C1:GUS (15 %); C2:GUS (13 %); V1:GUSΔC (9.7 %); C3:GUS (5.7 %); V1:GUS (2.8 %); V2:GUS (not applicable).

1.9.2.3 The TLCV promoter:GUS fusions are active in tobacco protoplasts

To assess whether the problematic generation of GUS–positive C2:GUS, V1:GUS and V2:GUS transgenic plants was related to chromosomal integration, transient infection of tobacco protoplasts with the full–length and truncated virion–sense promoter–GUS fusions was performed. Exceptionally high GUS expression was obtained with both the V1:GUS and V2:GUS constructs (approximately 200-fold over expression compared with expression from a CaMV 35S:GUS construct), attributed to Rep–mediated replication of the construct plasmids. Whilst GUS expression from the V1:GUSΔC (0.08 %) and V2:GUSΔC (0.39 %) constructs in protoplasts was relatively low, co–transfection of a TLCV dimeric (replication–competent) construct with the V2:GUSΔC vector increased expression *ca.* 30-fold. The authors suggested that the TLCV–mediated increase in expression indicated efficient V2 promoter activity in protoplasts required the C2 ORF gene product, TrAP, which was not coded for by the V1:GUSΔC and V2:GUSΔC constructs. A *ca.* 85% reduction in GUS expression from the V2:GUS construct following inactivation of the C2 ORF supported this hypothesis. Thus, it appeared that whilst the TLCV virion–sense promoters were active in protoplasts, stable introduction into plants abolished detectable transcription from these promoters and was only recovered following deletion of specific TLCV sequences.

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[1] This author's calculations

Dry and co-workers (2000) put forward two possible explanations. Firstly, the presence of cis–acting TrAP-sensitive repressor elements could be acting on virion–sense promoter activity. While a similar situation exists for TGMV TrAP–mediated CP promoter activity (see above), it would therefore follow that expression of TrAP from the TLCV V2:GUS construct would transactivate V2 promoter activity, at least in vascular tissue (see later). It is also of interest that whilst the TGMV repressor element was inactive in dedifferentiated tissue, this is not the case for the putative TLCV repressor element. The truncated TGMV CPpro:GUS (A75 construct, Sunter and Bisaro 1997), which lacked a functional C2 ORF, showed a lack of mesophyll tissue-specific activity in *N. benthamiana*. This is in contrast to the TLCV CPpro:GUS (V2:GUSΔC) construct, which directed constitutive expression in host tissues. Whether this discrepancy indicated a lack of a mesophyll–specific CPpro repressor in TLCV or the different availability of host transcription factors in mesophyll tissue between tobacco (TLCV V2:GUSΔC) and *N. benthamiana* (TGMV A75) remains undetermined.

Secondly, examination of chromosome–integrated C2:GUS, C3:GUS and V1:GUS constructs revealed the consistent mutation of the C1 ORF into a non–functional state, suggestive of Rep–mediated lethality in transgenic tobacco. In effect, each of the TLCV promoter:GUS constructs active in transgenic tobacco lacked a functional C1 ORF. Support for this hypothesis comes from the inability to stably introduce TLCV (A. Rezaian, unpublished observation) or TYLCV (Bendahmane and Gronenborn 1997) C1 expression constructs into tobacco.

1.9.2.4 The TLCV promoter:GUS fusions in tobacco tissue direct different patterns of GUS expression

In situ histochemical analysis of GUS activity in each TLCV promoter-GUS plant revealed three broad patterns of tissue–specific GUS expression. The C1:GUS, C4:GUS and

V2:GUSΔC constructs directed essentially constitutive expression throughout leaf and stem tobacco tissue, whilst the C2:GUS and C3:GUS constructs directed expression predominantly in root and leaf vascular tissues. The V1:GUSΔC construct directed what was termed reduced vascular expression, which was constitutive expression except in leaf and root vascular tissue. The differing expression patterns between the truncated virion–sense constructs is supportive of previous findings of two different virion–sense transcripts expressed during TLCV infection (Mullineaux et al. 1993).

The authors note that as GUS expression was only obtained with a truncated version of the V1 promoter construct, it remains unclear whether the reduced vascular expression pattern observed accurately represents V1 promoter activity *in planta*. The finding of a single bicistronic transcript for the C2 and C3 ORFs (Mullineaux et al. 1993) is also supported by the similar GUS expression patterns observed for the C2:GUS and C3:GUS constructs.

1.10 Virus-induced transcriptional gene silencing

Of relevance to the results and discussion presented in this thesis is the category of homology-dependent gene silencing known as virus-induced transcriptional gene silencing (VITGS). To date, the general paradigm for VITGS can be described as the silencing of a CaMV 35S promoter-driven plant transgene following infection by a wild-type or recombinant virus carrying a copy of the CaMV 35S promoter. The following section summarizes the reported incidences of VITGS, which have involved the caulimovirus *Cauliflower mosaic virus* (CaMV), the potexvirus *Potato virus X* (PVX) and the tobravirus *Tobacco rattle virus* (TRV).

1.10.1 *Cauliflower mosaic virus*

A number of *Brassica* species, including *B. napus*, exhibit a recovery phenotype following infection by CaMV, which is characterised by the development of new asymptomatic leaves approximately 50 days post infection (dpi). The recovery phenotype was attributed to a

pathogen-induced RNA silencing response by the host plant, which possibly caused CaMV to enter a post-replicative state in recovering tissue. Transgenes carrying CaMV sequences in *B. napus* were also silenced following CaMV infection (Al-Kaff et al. 1998).

GUS expression driven by a CaMV 35S promoter in CaMV-infected transgenic *B. napus* was silenced in leaf tissue by 50 dpi. Silencing of GUS expression was first associated with local lesions at the site of virus inoculation. Subsequently the silencing of GUS activity spread from interveinal tissue into mesophyll tissue until the entire leaf was silenced at 50 dpi. The analysis of transcription by nuclear run-on assay from the CaMV 35S:GUS transgene in tissue at 50 dpi revealed that silencing occurred at the level of transcription.

CaMV-induced transcriptional silencing of a 35S promoter-driven herbicide-resistance transgene in *B. napus* has been reported (Al-Kaff et al. 2000). Analysis of cytosine methylation of the CaMV 35S promoter sequence did not indicate a change in methylation following silencing. Thus infection by the DNA virus CaMV resulted in the silencing of homologous nuclear promoter sequences but was not associated with changes in cytosine methylation of the promoter sequence.

1.10.2 *Potato virus X*

Expression from a CaMV 35S promoter:GFP transgene in transgenic *N. benthamiana* was silenced following infection by recombinant PVX carrying a 347-nt fragment of the CaMV 35S promoter (Jones et al. 1999), indicating that RNA viruses are capable of inducing VITGS. Systemically infected leaves were silenced for GFP expression and correlated with a significant reduction of GFP mRNA. However, replication of the recombinant PVX did not appear to be significantly affected in the GFP-silenced tissue, consistent with the lack of recovery in hosts from PVX infection. Analysis of cytosine methylation of the CaMV 35S promoter sequence in silenced leaves by methylation–sensitive restriction enzyme digestion

showed that hypermethylation occurred within and upstream of the promoter sequence, but did not extend into the transcribed (i.e. GFP) transgene sequence. Thus, VITGS by an RNA virus correlated with the sequence-specific hypermethylation of the homologous nuclear sequences.

1.10.3 *Tobacco rattle virus*

Transgenic *N. benthamiana* carrying a CaMV 35S promoter:GFP transgene was infected with recombinant TRV carrying the 347-nt CaMV 35S promoter fragment. In systemically infected leaves, GFP expression was silenced at the transcriptional level and correlated with a 40 to 50-fold increase in the methylation of restriction sites containing symmetric and asymmetric cytosines in the CaMV 35S promoter sequence (Jones et al. 2001). Seeds collected from GFP-silenced flowers were germinated and the resultant progeny tested for GFP expression. GFP was silenced in the progeny plants (which were virus-free) and correlated with hypermethylation of symmetric cytosines in the CaMV 35S promoter sequence.

Transgene expression in progeny from a silenced parent was restored during further plant growth in approximately 70 % of plants. The CaMV 35S promoter sequence in the progeny that retained silencing was hypermethylated at symmetric cytosines only. The role of the methyltransferase METI in the maintenance of silencing in the progeny was tested by TRV-induced gene silencing of METI transcription. METI silencing resulted in the release of GFP silencing in progeny and correlated with hypomethylation of the CaMV 35S promoter sequence. The heritable, yet reversible silencing of the CaMV 35S promoter in progeny indicated that VITGS is likely to be the result of a type of epimutation. Furthermore, the silencing of the CaMV 35S promoter was associated with changes in DNA methylation which were inherited by silenced progeny.

1.11 Scope of this thesis

This work is part of a broader joint research program at the University of Adelaide and CSIRO Plant Industry aimed at understanding the molecular biology of TLCV and developing molecular control strategies against this important geminivirus. Effective measures to control TLCV, and geminiviruses generally, have not been developed. An expanding area of plant-microbe interaction research is the adaptive mechanism known as homology-dependent gene silencing. Characterisation of the HdGS response of plants to TLCV infection may advance efforts to control this virus. Within this framework the specific objectives of the work described in this thesis include:

1. Characterising the parameters for the silencing of TLCV promoter:GUS transgenes in transgenic tobacco following TLCV infection.
2. Characterising the biochemical changes occurring during the silencing of the TLCV promoter:GUS transgenes in tobacco following TLCV infection.
3. The analysis of transgene expression in progeny derived from non-silenced and silenced TLCV promoter:GUS plants.
4. The characterisation of small interfering RNA accumulation during TLCV infection as a possible mediator of the TLCV promoter:GUS transgene silencing.

Chapter 2

General Materials and Methods

2.1 Materials

2.1.1 Solutions

All chemicals used were analytical or molecular biology grade. Solutions were prepared with ultra-pure water and autoclaved where appropriate. The compositions of buffers and growth media are outlined in Table 2.1. Equilibrated liquid phenol was obtained from USB (Cleveland, USA).

Table 2.1 Solutions and their compositions

Solution	Composition
Sample loading buffers	
Agarose-gel loading buffer (10 X)	78% glycerol, 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol, 10.0 mM EDTA
Formamide loading buffer	Deionized formamide, 0.1% (w/v) bromophenol blue, 0.1 % (w/v) xylene cyanol
Extraction buffers	
DNA extraction buffer	50 mM Tris-HCl pH 8.0, 100 mM NaCl, 10 mM EDTA, 1 % (v/v) β-mercaptoethanol, 0.1% (v/v) SDS
RNA extraction buffer	50 mM Tris-HCl pH 8.0, 100 mM NaCl, 5 mM EDTA, 2 % (v/v) β-mercaptoethanol, 0.1% (v/v) SDS
Electrophoresis buffers	
Agarose gel electrophoresis buffer (1 X TBE)	90 mM Tris-borate pH 8.3, 2 mM EDTA
Growth media	
LB broth	1% (w/v) bacto-tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl, pH 7.0. To prepare solid medium 1.2% bacto-agar was added
Other solutions	
TE buffer	10 mM Tris-HCl pH 8.0, 1 mM EDTA
1 X SSC	150 mM NaCl, 15 mM trisodium citrate
Hybridization buffer	0.25 M Na_2HPO_4, 7 % (v/v) SDS, 1 mM EDTA

2.1.2 Oligonucleotides

Synthetic oligodeoxyribonucleotides (oligonucleotides) were obtained from GeneWorks, Adelaide, South Australia. The sequences of the oligonucleotides used in this study are listed in Table 2.2.

Table 2.2 Oligonucleotide primers used in this study

Primers	Size (nt)	Nucleotide positions[a]	Sequences from 5' to 3'[b]	Underlined restriction site
GUS1286-1305[s]	20	1286-1305	AAGCAACGCGTAAACTCGAC	
GUS1766-1785[as]	20	1766-1785	TTTTTCACCGAAGTTCATGC	
Rub1072-1091[s]	20	1072-1091	CTACCGCATCGAGCGTGTTG	
Rub1393-1412[as]	20	1393-1412	CAAGTCCACCGCGAAGACAT	
TLCVfor[s c]	30	39-68	TTTTTTACGTGATTGATGTGATCTGTCGAC	
GUSrev[as c]	31	127-157	CATTAAAACTACCTAACACAACAATTACCCA	
TLCV39-68[s]	30	39-68	CCCCCCACGTGATTGATGTGACCTGTCGAC	
GUS127-157[as]	31	127-157	CGTTAAAACTGCCTGGCACAGCAATTGCCCG	
5Sfor[s d]	42		TAAGAAAATCTAGAGTGTAAGGAATGTTGGATGCGATTATAT	
5Srev[as d]	44		TTCATTAATACAAGCTTTACCAAAAAAAAAAATACAACA CGAAA	
TLCV2647-2676[s]	30	2647-2676	ttaAGATCTGTAAATGAATCGGTGTCTGGG	BglII
TLCV124-143[as]	28	124-143	agaAGATCTGGGCCTAAATACTTAGGGC	BglII
TLCV2758-11[as]	21	2758-11	CGGTAATATTAGACGGATGGC	
T7	20		TAATACGACTCACTATAGGG	
SP6	20		ATTTAGGTGACACTATAGAA	

[a]Nucleotide positions as denoted in the β-glucuronidase (GUS) ORF sequence (AF485783), Rubisco large subunit (Rub) ORF sequence (Shinozaki and Sugiura 1982) and *Tomato leaf curl virus* (TLCV) sequence (Dry et al. 1993)
[b]Lowercase letters indicate extra nucleotide residues containing restriction sites introduced for cloning
[c]Primers designed for amplifying bisulfite modified TLCV and GUS DNA
[d]Primers designed by Fulnecek et al. (1998) for amplifying bisulfite modified 5S rDNA sequences from tobacco
[s, as]Denote sense- and antisense-strand, respectively

2.1.3 Bacterial strains and plasmid vectors

Escherichia coli strain DH5α (Stratagene, USA) was used for routine cloning work. *Agrobacterium tumefaciens* strain C58 was used for agroinoculation of geminivirus constructs.

Routine cloning was carried out using the vector pGEM-T-Easy (Stratagene, Australia), which carries ampicillin resistance and promoter sequences for T7 and SP6 RNA polymerases flanking the multiple cloning site. The linearised vector as supplied by the manufacturer contains a 3' terminal thymidine at either end of the vector to allow ligation to PCR products

generated by compatible DNA polymerases such as *Taq*. Screening of DNA inserts successfully ligated into pGEM-T-Easy was performed by growing *E. coli* DH5α transformed (see section 2.2.6) with ligation mixtures of DNA inserts and pGEM-T-Easy (see section 2.2.5) on solid LB media (see Table 2.1) containing ampicillin (50 μg/ml), X-gal (56 μg/ml) and IPTG (18.75 μg/ml).

The binary vector pBin19 (Bevan 1984) carrying kanamycin resistance was used as a vector for *A. tumefaciens*-mediated agroinoculation.

2.1.4 Transgenic plant lines

A series of six transgenic *N. tabacum* (cv. Samsun) lines carrying stably integrated TLCV promoter:GUS transgenes were generated by Dry and co-workers (2000). These transgenes, with the exception of the C1:GUS construct, were in-frame N-terminal translational fusions of an *E. coli uidA*-NOS terminator cassette with the viral ORFs and directed expression of a GUS protein with a leader sequence of 1-13 amino acids of virus gene product. To construct the C1:GUS transgene, a *uidA*-NOSter cassette was inserted in the 5'-untranslated region of the C1 ORF, 24 bp upstream of the C1 start codon. Unless stated otherwise, all transgenic tobacco plants used were T_1 generation. These were plants were generated by the self-fertilisation of T_0 parents carrying multiple transgene inserts.

2.2 Methods

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

2.2.1 Restriction enzyme digestion of DNA

DNA was digested with restriction endonucleases from Boehringer Mannheim (Germany), Promega (USA), Roche (Switzerland) or Fermentas (USA) using buffer systems supplied by the manufacturer.

2.2.2 Gel electrophoresis

2.2.2.1 Agarose gel electrophoresis

Agarose gels were prepared from 0.7-2.0% (w/v) solutions of Type I-A: Low EEO agarose containing 0.5 μg/ml (w/v) ethidium bromide in 1 X TBE buffer (Table 2.1). Horizontal minigel systems (EasyCast Electrophoresis Systems, Models B1A or B2, OWL Scientific, Inc., Cambridge) were used. DNA samples were adjusted to 1 x loading buffer (Table 2.1) before applying to the wells. Gibco 1 Kb Plus DNA markers were used as a low range molecular weight marker. Gels were electrophoresed in 1 X TBE running buffer and photographed using a short wavelength UV transilluminator.

2.2.2.2 Polyacrylamide gel electrophoresis

Denaturing polyacrylamide gels for RNA analysis contained 15 % (w/v) acrylamide, 1 x TBE and 7 M urea. Polymerization was initiated by the addition of 0.6 mg ml^{-1} freshly prepared ammonium persulfate and 0.5 μl of TEMED per 1 ml of solution. The solution was cast between plates (90 x 70 x 1 mm) suitable for use in the Mini-PROTEAN II electrophoresis module (BioRad, USA) and allowed to set for at least 30 min. The gel wells were rinsed several times with 1 X TBE to minimize urea accumulation in the wells. RNA samples adjusted to a minimum of 0.4 X formamide loading buffer (Table 2.1) and denatured by heating at 95°C for 2 min before applying to the wells. RNA samples were electrophoresed at 25-30 mA in 1 X TBE until the bromophenol blue dye band had reached the bottom of the gel

casting plates. Gels were soaked in a solution of 1 X TBE containing 2 μg ml^{-1} ethidium bromide for 15 min with gentle agitation and the nucleic acids photographed using a short wavelength UV transilluminator.

2.2.3 Purification of DNA from agarose gel slices

DNA bands excised from agarose gels after staining with ethidium bromide were extracted from the gel using a QIAquick gel extraction kit (QIAGEN) according to the manufacturer's instructions.

2.2.4 DNA amplification

Polymerase chain reactions (PCR) were carried out in a volume of either 20 or 50 μl containing 1 X reaction buffer (Invitrogen), DNA template, oligonucleotide primers (each at 0.5 μM), 200 μM each of dCTP, dGTP, dATP and dTTP, 1.5 mM $MgCl_2$ and 0.5 U of Taq or Platinum Taq DNA polymerase (Invitrogen). The mixture was subjected to a 30 cycle PCR program consisting of 30 sec at 94°C, 1 minute at 55°C and 1 minute at 72°C, preceded by a single step at 95°C for 1 minute. When necessary, the PCR products were purified with a QIAquick PCR purification kit (QIAGEN) according to the manufacturer's instructions.

2.2.5 DNA ligation

DNA purified by agarose gel fractionation (see section 2.2.3) was ligated to vector DNA in a molar ratio of approximately 3:1, respectively. The ligation was carried out in a volume of 50 μl containing DNA insert, vector DNA, ligation buffer (Boehringer Mannheim) and 5 U of T4 DNA ligase (Boehringer Mannheim). The reaction was incubated overnight at 4°C and as required, 1-5 μl of ligation mixture was used for electroporation.

Ligations of gel-purified DNA inserts into pGEM-T-Easy vector were performed according to the manufacturer's instructions, using 3 μl of DNA insert to 1 μl of supplied vector. The

reaction was incubated overnight at 4°C and ethanol precipitated (see section 2.2.15). Typically, pellets were resuspended in 15 µl 10 mM Tris-HCl pH 8.0 and 10 µl used for electroporation.

2.2.6 Transformation of bacteria with recombinant plasmids

Transformation of bacteria with plasmids was done by electroporation, using a Gene-Pulser apparatus (BioRad, USA). Electrocompetent cells were prepared according to the instructions accompanying the Gene-Pulser apparatus.

Plasmid DNA for electroporation was mixed with 25-50 µl of *E. coli* electrocompetent cells and transferred to an ice-cold cuvette (Bio-Rad or Invitrogen) with a 0.1 cm electrode gap. The cuvette was placed into the Gene-Pulser electroporator set at 1.8 kV, 25 µFD and 200 Ohm and given a single pulse immediately. The cells were resuspended in 700-1000 µl LB media and incubated at 37°C for one hour. The culture was centrifuged at 5,000 *g* for 5 min and the supernatant decanted. The pellet was resuspended in 200-400 µl of LB broth and spread onto a 1.2 % LB agar plate containing appropriate antibiotics and the plate cultures incubated overnight at 37°C.

2.2.7 Growth of bacteria

Liquid cultures were set up by inoculating LB broth with a single bacterial colony or one loopful of frozen bacterial glycerol stock. Cultures were incubated at 37°C (*E. coli)* or 28°C (*A. tumefaciens*) overnight with shaking. As appropriate, the growth media contained the antibiotics ampicillin (100 µg/ml), kanamycin (50 µg/ml) or rifampicin (25 µg/ml).

2.2.8 Preparation of bacterial glycerol stocks

Where required, glycerol stocks of *E. coli* or *A. tumefaciens* were prepared by adding 1 ml of sterile 40% glycerol to 1 ml of overnight culture, snap-freezing in liquid nitrogen and storing at -70°C.

2.2.9 Preparation of bacterial plasmid DNA

Minipreparation of plasmid DNA was performed using a QIAprep Spin Miniprep Kit (QIAGEN) according to the manufacturer's instructions. Large scale (20-50 ml cultures) preparation of plasmid DNA was carried out using the QIAGEN Plasmid Midi-kit (QIAGEN) according to the manufacturer's instructions.

2.2.10 DNA sequencing

The dideoxynucleotide chain termination sequencing method was used to sequence DNA using the BigDye Terminator Cycle Sequencing Ready Reaction Kit (version 2 or 3) according to the manufacturer's instructions (Applied Biosystems, England). Sequencing reactions consisted of 8 µl of Terminator Ready Reaction Mix, 3.2 pmol DNA oligonucleotide primer, DNA template (200-500 ng dsDNA or 30-90 ng PCR-generated DNA) and deionized water to 20 µl. The assembled reactions were vortexed and briefly centrifuged. Thermal cycling was as follows: 25 cycles of 96°C for 10 s, 50°C for 5 s and 60°C for 4 min. The reactions were held at 4°C until purification. The reactions were transferred to a 1.5 ml Eppendorf tube and 80 µl of 75 % isopropanol added. The reactions were vortexed briefly and incubated at room temperature for 15 min before centrifugation in a microcentrifuge at 16,700 *g* for 20 min. The pellets were washed with 250 µl 75 % isopropanol by vortexing briefly and centrifuged as above for 5 min. The supernatant was discarded and the pellets dried under vacuum for 10 min. Analysis of the sequencing products

was done at the Institute of Medical and Veterinary Science, Adelaide, South Australia using an ABI PRISM system (Applied Biosystems, England).

2.2.11 Preparation of ^{32}P-labelled probes

2.2.11.1 Labelling of DNA

^{32}P-labelled probes were synthesized by random priming using a *redi*prime II kit (Amersham Biosciences, England) according to the manufacturer's instructions. 2.5-25 ng of template DNA was heated at 95°C for 5 min, cooled on ice for 5 min and labelled with 5 µl of Redivue stabilised [α-^{32}P]dCTP (3000 Ci/mmol; Amersham Biosciences, England) at 37°C for 10-30 minutes. Unincorporated dNTPs were removed by passing the probe through a ProbeQuant G-50 Micro Column (Amersham Biosciences, Australia) according to the manufacturer's instructions. As required, the radioactivity of the probe was quantified using a Beckman LS3801 liquid scintillation counter. DNA probes were denatured by heating at 95°C for 10 min before use.

2.2.11.2 Labelling of RNA

^{32}P-labelled RNA was synthesized by incorporation of [α-^{32}P]UTP during *in vitro* transcription reactions. The DNA template for the RNA transcription reaction consisted of the desired DNA sequence inserted into the pGEM-T-Easy vector, which was then linearised immediately downstream of the 3'-end of the insert sequence by restriction digestion (see section 2.2.1). The digestion reaction was fractionated by agarose gel electrophoresis (typically 1 % agarose, see section 2.2.2.1), the DNA extracted (see section 2.2.3), quantified (see section 2.2.16) and 0.2 µg used in each transcription reaction.

Each 20 µl RNA transcription reaction consisted of 1 x transcription buffer (Promega, USA), 10 mM DTT, 20 U Superase.In (Ambion, USA), 500 nM each of ATP, GTP and CTP, 12 nM

UTP, 4 µl of [α-^{32}P]UTP (3000 Ci/mmol; PerkinElmer, USA) and 10 U T7 RNA polymerase (Promega). As required, the transcription buffer and RNA polymerase was replaced with 1 x SP6 transcription buffer and 10 U SP6 RNA polymerase (Ambion, USA). The reaction was incubated at 37°C for 1 hour, 1 U RQ1 DNase I (Promega) added and the reaction incubated at 37°C for a further 10 min. The RNA was precipitated by adding 15 µl of 5 M ammonium acetate, mixing by pipetting, and then adding 75 µl ethanol and mixing by pipetting. The reaction was incubated on ice for 10 min and centrifuged at 13, 250 *g* for 15 min. The pellet was resuspended in 100 µl TE buffer (Table 2.1) before the addition of 75 µl 5 M ammonium acetate. After mixing by pipetting, 375 µl ethanol was added, the solution mixed again by pipetting and incubated on ice for 10 min before centrifuging as above. The pellet was air-dried for 5 min and resuspended in 20 or 40 µl TE buffer (Table 2.1).

The RNA probe was partially hydrolysed by the addition of 300 µl of freshly prepared 200 mM sodium carbonate (80 mM $NaHCO_3$, 120 mM Na_2CO_3) followed by incubation at 60°C (Wang et al. 2001). RNA was hydrolysed to a specific average size by varying the incubation time according to the formula: $t = (L_i - L_f)/(k \times L_i \times L_f)$, where t = time in min, L_i = initial length of the probe in kilobases, L_f = final length of the probe in kilobases and k = rate constant of 0.11 kb/min. The hydrolysis reaction was terminated by the addition of 20 µl of 3 M sodium acetate (pH 5.2).

2.2.12 DNA extraction

DNA was extracted from leaf tissue. Small, expanding leaves were generally chosen for DNA extraction, however when larger leaves were used the mid-rib was first removed and discarded. The mid-rib was not discarded for DNA extractions of samples to be used for the analysis of virus DNA content. Leaf tissue of known weight was powdered in liquid nitrogen and mixed with three volumes of DNA extraction buffer (Table 2.1). The mixture was

incubated with gentle agitation for 15-30 min and then extracted three times with an equal volume of equilibrated phenol and once with an equal volume of chloroform. Alternatively, phenol:chloroform:isoamylalcohol (25:24:1) and chloroform:isoamylalcohol (24:1) was substituted for phenol and chloroform, respectively. The resulting supernatant was ethanol precipitated (see section 2.2.15) and resuspended in TE buffer (Table 2.1). The suspension was incubated with 1 $\mu g\ \mu l^{-1}$ ribonuclease A at 37°C for one h and the DNA recovered by phenol:chloroform:isoamylalcohol extraction and ethanol precipitation. The DNA pellet was resuspended in 10 mM Tris-HCl pH 8.0.

2.2.13 Total RNA extraction

Total RNA extraction was typically performed on leaf tissue. Small, expanding leaves were generally chosen for DNA extraction, however when larger leaves were used the mid-rib was first removed and discarded. The mid-rib was not discarded for RNA extractions of samples to be used for the analysis of virus RNA content. Leaf tissue of known weight was powdered in liquid nitrogen and mixed with three volumes of RNA extraction buffer (Table 2.1) and three volumes of equilibrated phenol (see section 2.1.1). The mixture was agitated vigorously for 10 min and centrifuged at either 3,920 *g* (for tissue amounts of more than 1 gram) in a bench-top centrifuge, or 16,700 *g* (for tissue amounts of 1 gram or less) in a microcentrifuge for 10 min. The supernatant was extracted twice with phenol and once with chloroform and the RNA recovered by ethanol precipitation (see section 2.2.15). The RNA pellet was resuspended in either deionized water or 10 mM Tris-HCl pH 8.0.

2.2.13.1 Total RNA extraction with Trizol LS Reagent

Total RNA suitable for short interfering RNA analysis was isolated using Trizol LS Reagent (Invitrogen, USA) according to the manufacturer's instructions with some modifications. Leaf tissue (typically 100 mg) was powdered in liquid nitrogen and added to 3 volumes of Trizol

LS Reagent. The resuspension was centrifuged at 12, 000 *g* for 10 min at 4°C in a bench-top centrifuge, the pellet discarded and 200 µl of chloroform added per 750 µl Trizol LS Reagent used for the initial resuspension. The mixture was shaken vigorously by hand for 15 s and incubated at room temperature for 2 min before centrifuging as above. The RNA was precipitated from the resulting supernatant by the addition of 250 µl 100 % isopropanol and 250 µl 1.2 M NaCl per 750 µl of Trizol LS Reagent used for the initial resuspension. The mixture was incubated at room temperature for 10 min, centrifuged as above and the RNA pellet washed with 400 µl 75 % ethanol by vortexing. The mixture was centrifuged at 7, 500 *g* for 5 min at 4°C in a bench-top centrifuge and the pellet air-dried for 5 min. Resuspension of the RNA pellet was in TE buffer (Table 2.1) (40 µl per 100 mg leaf tissue). Resuspension of the pellet was aided, if required, by vortexing and heating at 50°C for 10 min.

2.2.14 Phenol extraction of nucleic acids from enzymatic reactions

Nucleic acids in enzymatic reactions were recovered by adjusting the reaction volume to 300-500 µl with deionized water or TE buffer (Table 2.1) and adding an equal volume of phenol:chloroform:isoamylalcohol (25:24:1). The mixture was vortexed for 2 min and centrifuged in a microcentrifuge at 16, 700 *g* for 3 min. The supernatant was added to an equal volume of chloroform:isoamylalcohol (24:1) and vortexed and centrifuged as above. The supernatant was then ethanol precipitated (see section 2.2.15) and the nucleic acid pellet resuspended in 10 mM Tris-HCl pH 8.0.

2.2.15 Ethanol precipitation of nucleic acids

Solutions containing DNA and/or RNA were precipitated by the addition of $1/10^{th}$ volume of 3 M sodium acetate, pH 5.2 and 2.5 volumes of ethanol, followed by incubation on ice or at -20°C for a minimum of 15 min. The precipitate was recovered by centrifugation in a

microcentrifuge at 16,700 *g* for a minimum of 20 min. Pellets were washed with 70 % ethanol before drying in a vacuum and resuspended in either deionized water, 10 mM Tris-HCl pH 8.0 or TE buffer (see Table 2.1).

2.2.16 Quantitation of nucleic acids by optical density measurement

The concentration of nucleic acids in solution was quantified by measurement of absorbance at 260 nm. DNA solutions were diluted (typically 1 in 20) in TE buffer (see Table 2.1) and 20 µl of the dilution applied to a quartz cuvette (path length = 10 mm; Starna, Australia). Absorbance spectra between 220-320 nm were measured using a UV-1601 UV-visible spectrophotometer (Shimazdu, Australia). Baseline absorbance values were established using TE buffer. Nucleic acid concentration (mg ml^{-1}) was calculated by using the following formula: absorbance at 260 nm x dilution factor x 1/extinction coefficient. The extinction coefficient used for DNA and RNA was 20 and 25 g^{-1} cm^{-1} L, respectively.

2.2.17 Southern blot hybridization analysis

DNA samples were fractionated in agarose gels (typically 1 %, see section 2.2.2.1) and blotted onto a Zeta-Probe nylon membrane (BioRad) by a rapid downward transfer system (Schleicher & Schuell, USA) according to the manufacturer's instructions. The membrane was washed in 2 X SSC (see Table 2.1) for 5 min with gentle agitation and the DNA cross-linked to the membrane using a UV Stratalinker 1800 (1.2 x 10^5 microjoules/cm^2, Stratagene, USA). The membrane was air-dried and stored in a plastic resealable bag, or used immediately. Membranes were prehybridized by incubating with 5 ml of hybridization buffer (Table 2.1) at 65°C for a minimum of 30 min. The hybridization buffer was replaced with fresh buffer (5 ml) and the probe added (see section 2.2.11.1). Hybridizations were done overnight at 65°C with rotation in a mini hybridisation oven (Hybaid, UK). Membranes were washed twice with 2 X SSC, 0.1 % SDS for 5 min at 65°C and once with 0.1 X SSC, 0.1 %

SDS for 20 min at 65°C. Membranes were wrapped in plastic wrap and exposed to Biomax film (Kodak, USA) at -70°C in a film cassette containing a Biomax intensifying screen (Kodak, USA).

2.2.18 Dot blot hybridisation analysis

Systemic virus infection was determined by DNA dot blot hybridisation analysis. Leaf tissue from agroinoculated plants (see section 2.2.20) was homogenised in two volumes of 0.5 M NaOH and centrifuged at 15,000 *g* for 30 min. Supernatant (4 µl) was dotted onto Zeta-Probe nylon membrane (BioRad). Membranes were briefly rinsed in chloroform, washed in 2 X SSC and UV cross-linked (see section 2.2.17). Membranes were hybridised overnight (see section 2.2.17) with random primer-generated ^{32}P-labelled DNA (see section 2.2.11.1) using full-length TLCV sequence as a template.

2.2.19 Growth of plants

Tobacco (*N. tabacum* var. Samsun), tomato (*Lycopersicon esculentum* var. Grosse Lisse) and datura (*Datura stramonium*) were maintained in glasshouses under containment conditions at 25 ± 3° C under natural lighting. For the collection of tobacco seeds, flowering tobacco plants were allowed to self-fertilise and the resulting seed pods harvested. Seeds were extracted from the seed pods and stored in polycarbonate tubes in the dark at room temperature.

2.2.20 Virus inoculation

Agroinoculation of plants at the 4- to 6-leaf stage was carried out by injecting 10 µl of *A. tumefaciens* strain C58 cultures carrying infectious multimeric head-to-tail copies of either TLCV, *African cassava mosaic virus* (ACMV), *Tomato yellow leaf curl Sardinia virus* (TYLCSV) (Dry et al. 1997) or TLCV-D1 (Behjatnia et al. 1996) genomic DNA inserted in pBIN19, into multiple sites along the main stem of the plants. Injection of cultures were done

using a 50 µl syringe with a 0.72 mm diameter needle (Model 705, Hamilton, Reno, Nevada) attached to a PB600 repeating dispenser (Hamilton, Reno, Nevada) allowing the delivery of 1 µl aliquots.

2.2.21 Analysis of GUS activity

Histochemical detection of GUS activity was done (Jefferson et al. 1986) as described by Dry et al. (2000). GUS assays of *in vitro* germinated seedlings were performed up to 10 days post germination without tissue dissection.

2.2.22 Reverse transcription-polymerase chain reaction

Reverse transcription-polymerase chain reactions (RT-PCR) were done using a SuperScript One-Step RT-PCR kit (Invitrogen) according to the manufacturer's instructions. RT-PCR was performed with 2 µg of total RNA extracted from leaf tissue (see section 2.2.13) pooled from multiple non-infected or TLCV-infected plants, with the addition of a DNase digestion step. DNase digestion reactions consisted of 1 X reaction buffer (40 mM Tris-HCl pH 8.0, 10 mM $MgSO_4$, 1 mM $CaCl_2$), 20 U DNase I (Promega, USA), 100 U of Superase.In (Ambion, USA) and 40 µg of RNA. Digests were done at 25°C overnight and the RNA recovered by phenol:chloroform:isoamylalcohol extraction (see section 2.2.14) and ethanol extraction (see section 2.2.15). Specific primers used were: GUS1286-1305, GUS1766-1785, Rub1072-1091 and Rub1393-1412 (see Table 2.2). RT-PCR products (5-20µl) were blotted to nylon membrane (see section 2.2.17) and probed with random primer-generated ^{32}P-labelled GUS DNA (see section 2.2.11.1).

Chapter 3

Virus-induced transcriptional gene silencing associated with TLCV infection

3.1 Introduction

Expression directed by geminivirus CP promoters can be regulated by virus factors. GUS expression in transgenic *N. benthamiana* carrying a full-length copy of TGMV DNA A (with a non-functional C2 ORF) with a partial replacement of the CP by GUS was not detectable until the C2 gene product was supplied *in trans* by TGMV infection (Sunter and Bisaro 1997). In a separate experiment, a truncated copy of the *Pepper huasteco yellow vein virus* (PHYVV, formerly known as *Pepper huasteco virus*) DNA A directed vascular-specific GUS expression from the CP promoter in tobacco (Ruiz-Medrano et al. 1999). Following the supply of PHYVV C2 gene product *in trans* by virus infection, GUS was expressed in both vascular and mesophyll tissue. Thus, geminivirus factors can transactivate the tissue-specific transcription patterns directed by integrated geminivirus promoter sequences.

The V2:GUSΔC transgene, which carries a truncated copy of the TLCV genome with GUS as a translational fusion with the CP ORF, directs constitutive GUS expression in tobacco tissues (Dry et al. 2000). The V2:GUSΔC transgene does not require the TLCV C2 gene product for activity. This is in contrast to similar TGMV- (Sunter and Bisaro 1997) and PHYVV-based (Ruiz-Medrano et al. 1999) transgenes, which require the TGMV and PHYVV virus C2 gene products, respectively, for constitutive GUS expression.

To resolve whether TLCV gene products supplied *in trans* by virus infection caused the alteration of tissue-specific transcription from integrated TLCV promoters, TLCV promoter:GUS tobacco plants were infected with TLCV (I. Dry, unpublished data). Systemic virus infection of the transgenic plants resulted in the silencing of GUS expression. The aim of the work described in this chapter was to characterise the TLCV-induced silencing of the TLCV promoter:GUS transgenes. Silencing of the V2:GUSΔC transgene was shown to be specific to TLCV infection and to occur in the leaf, stem and floral tissue of the plants.

Furthermore, silencing was independent of the TLCV C3, C4 and V1 gene products and was due to inactivation of the integrated V2:GUSΔC promoter.

3.2 Materials and methods

3.2.1 Analysis of GUS activity in TLCV promoter:GUS plants following TLCV infection

TLCV promoter:GUS T_1 (see section 2.1.4) seeds were germinated and maintained under containment conditions in a glasshouse. The leaves of seedlings at the 4-leaf stage were assayed for GUS activity (see section 2.2.21) and the plants not positive for GUS activity were discarded.

The TLCV promoter:GUS plants were inoculated at the 6-leaf stage with a 24-48 h culture of *A. tumefaciens* carrying copies of the full-length TLCV sequence inserted into a binary pBIN19 vector (see 2.2.20).

The small expanding leaves of each plant at 50 days post inoculation (dpi) were assayed for GUS activity (see section 2.2.21). The small expanding leaves of the TLCV promoter:GUS plants inoculated with *A. tumefaciens* were assayed at 50 dpi by dot blot hybridisation for TLCV infection (see section 2.2.18).

The small expanding leaves from V2:GUSΔC plants inoculated with TLCV (see section 2.2.20) were analysed for GUS activity (see section 2.2.21) at various times between 0 and 50 dpi. The plants were maintained in the glasshouse until the development of preanthesis floral (unopened flower bud) tissue. Sections of stem tissue and preanthesis floral tissue were analysed for GUS activity (see section 2.2.21).

3.2.2 Dot blot hybridisation analysis of V2:GUSΔC plants inoculated with *African cassava mosaic virus* and *Tomato yellow leaf curl Sardinia virus*

Multiple plants (2-3) at approximately the six-leaf stage and positive for GUS activity were inoculated with either TLCV, the D1 strain of TLCV (TLCV-D1) (Behjatnia et al. 1996), ACMV, TYLCSV (Dry et al. 1993) or mock-inoculated (using *A. tumefaciens* carrying a pBIN19 binary vector, which did not contain an insert) (see section 2.2.20).

The plants were analysed for GUS activity at 50 dpi. Virus infection at 50 dpi was analysed by dot blot hybridisation (see section 2.2.18) with the following modification. TLCV and TLCV-D1 infection in inoculated V2:GUSΔC plants was analysed by dot blot hybridisation using a probe generated from full-length TLCV DNA. For ACMV and TYLCSV probes, the DNA templates were PCR products amplified from cloned ACMV or TYLCSV genome sequences. The PCR products corresponded to the C1 ORF of either virus and were supplied by Dr Baochuan Lin. The ^{32}P-labelling reaction was done using 1 µl of a 20 µl PCR reaction product.

The percent nucleotide identity of the intergenic region and 5'-end of the C1 ORF sequences (nucleotide positions 2354-149) of TLCV to either ACMV or TYLCSV was calculated using the GAP program (ANGIS; http://www.angis.org.au/WebANGIS).

3.2.3 Analysis of GUS activity in V2:GUSΔC plants inoculated with TLCV carrying a C3 ORF knockout

In previous work to ascertain the function of the various TLCV gene products, a series of mutations were introduced into the infectious TLCV binary vector used for viral inoculation. A C3 ORF-mutant (C3mut) was produced by a nucleotide mutation at TLCV co-ordinate 1418 to create a premature stop codon (Rigden et al. 1996).

Six V2:GUSΔC plants that tested positive for GUS activity were inoculated with the TLCV C3mut construct (see section 2.2.20). Small expanding leaves were tested at 50 dpi for GUS activity (see section 2.2.21) and virus infection by dot blot hybridisation using a probe generated from full-length TLCV DNA (see section 2.2.18).

3.2.4 Analysis of GUS activity in V2:GUSΔC plants inoculated with TLCV via graft transmission

A wild tomato plant showing symptoms associated with TLCV infection was collected from the Lakeland district of north Queensland, Australia. The plant was tested by dot blot hybridisation analysis and shown to be infected with TLCV.

Scions were taken from the infected tomato plant and grafted onto five V2:GUSΔC rootstocks that were positive for GUS activity. The V2:GUSΔC rootstocks were tested for GUS activity (see section 2.2.21) and TLCV infection (see section 2.2.18) at 50 days post grafting.

3.2.5 RT-PCR analysis of GUS transcription in TLCV-infected C1:GUS and V2:GUSΔC plants

Total RNA was extracted from pooled tissue harvested from either five non-silenced or five silenced C1:GUS or V2:GUSΔC plants. RT-PCR reactions were done (2.2.22) specific for a 499 bp GUS product (Table 2.2; GUS1286-1305 and GUS1766-1785 primers) or a 340 bp tobacco Rubisco product (Table 2.2; Rub1072-1091 and Rub1393-1412 primers). As a control, RT-PCR was done with GUS-specific primers but without the reverse transcriptase. The PCR products were analysed by Southern blotting (see section 2.2.17) and probed for GUS-specific cDNA using a full-length GUS ORF DNA template.

3.2.6 Nuclear run-on analysis of GUS transcription in TLCV-infected V2:GUSΔC plants

3.2.6.1 Isolation of nuclei from tobacco leaf

V2:GUSΔC plants were placed in the dark for 24 h prior to extraction. Small expanding leaves were harvested from five non-silenced or five silenced (i.e. TLCV-infected) plants, the mid-rib removed and the tissue pooled.

One g of tissue was chopped manually over ice for 20 min in 1 ml of buffer A (0.44 M sucrose, 2.5 % (w/v) Ficoll 400, 5.0 % (w/v) Dextran T40, 25 mM Tris-HCl pH 8.5, 10 mM $MgCl_2$, 2.5 % (v/v) Triton X-100, 10 mM β-mercaptoethanol, 2 mM spermine) (Inze et al. 1992). The chopped tissue was diluted with another 9 ml of buffer A, filtered through one layer of cheesecloth and two layers of Miracloth (Calbiochem) and gently agitated by hand for 30 s. The filtrate was centrifuged at 1,000 *g* for 30 min at 4°C and crude nuclear pellets resuspended in 250 µl of buffer B (0.44 M sucrose, 2.5 % (w/v) Ficoll 400, 5 % (w/v) Dextran T40, 25 mM Tris-HCl pH 8.5, 10 mM $MgCl_2$, 2.5 % (v/v) Triton X-100, 10 mM β-mercaptoethanol). The resuspension was loaded onto a 2 ml 60 % sucrose column (column height = 1.75 cm) and centrifuged at 1,000 *g* for 30 min at 4°C. The pellet was resuspended in 250 µl of buffer C (50 mM Tris-HCl pH 8.5, 5 mM $MgCl_2$, 10 mM β-mercaptoethanol, 20 % (v/v) glycerol), snap-frozen in liquid nitrogen and stored at -80°C.

The concentration of nuclei was determined using a haemocytometer under a fluorescence microscope after staining the nuclei with either 4',6-diamidino-2-phenylindole or SYBR Gold (Molecular Probes).

3.2.6.2 Nuclear run-on transcription

A modified transcription protocol was developed based on previously described methods (Dehio and Schell 1994; Santoso and Thornburg 1998; Ye and Signer 1996). V2:GUSΔC nuclei (3.5 x 10^6) were pelleted at 4°C for 5 min at 1,000 *g* and resuspended in 225 µl of reaction buffer (50 mM Tris-HCl pH 7.9, 0.1 mM DTT, 10 mM $MgCl_2$, 50 mM $(NH_4)_2SO_4$, 555 µg ml^{-1} BSA, 0.2 mM GTP, 0.2 mM ATP, 0.2 mM CTP and 175 U of the RNase inhibitor SUPERase.In (Ambion, USA)). To this was added 25 µl (250 µCi) of [α-^{32}P]UTP (3000 Ci $mmol^{-1}$; 37 MBq ml^{-1}; Perkin-Elmer, Australia) and the reactions incubated at 30°C for 2 h. RNase-free DNase (150 U) and 45µl DNase digestion buffer (Promega, USA) was added and incubation was continued for a further 10 min. Proteinase K (80 µg ml^{-1}), 100 µg ml^{-1} *E. coli* tRNA and 50 µl proteinase digestion buffer (100 mM Tris-HCl pH 7.5, 50 mM EDTA, 10 % (v/v) SDS) was added and the reactions incubated at room temperature for 25 min. Nucleic acids were purified by phenol extraction, chloroform extraction (see section 2.2.14) and passed through Micro Bio-Spin P30 Tris columns (BioRad, USA) according to the manufacturer's instructions. Eluates were pooled and 1 µl aliquots counted in a scintillation counter.

3.2.6.3 Preparation of DNA filters and hybridisation

Purified DNA (0.5-5 µg) was bound to Zeta-Probe GT nylon membranes (BioRad) using a Hoefer PR 648 slot-blot apparatus according to the membrane manufacturer's instructions (BioRad). Hybridisations were done in 0.25 M sodium phosphate (pH 7.2), 7 % (v/v) SDS at 65°C for 72 h using all of the ^{32}P-labelled RNA. Hybridized filters were washed at 65°C in 2 X SSC, 0.1 % SDS for 2 x 5 min, then in 0.1 X SSC, 0.1 % SDS for 20 min and exposed to Kodak Biomax film with an intensifying screen at -70°C for 12-72 h.

3.2.7 Wholemount *in situ* hybridisation analysis of TLCV infection in V2:GUSΔC plants

A modified version of the wholemount *in* situ hybridisation method described by Al-Kaff and Covey (1996) was used. Small expanding leaves of approximately 3-5 cm length were harvested from non-infected or infected V2:GUSΔC plants and the mesophyll tissue from one or both sides of the leaves discarded. The leaves were then incubated overnight in 100 % ethanol at 37°C. The leaves were incubated in 0.5 M NaOH for 15 min with gentle agitation and then washed twice in 1 M Tris-HCl (pH 8.0) for 10 min. After a 30 s wash in 2 X SSC buffer, the leaf DNA was cross-linked using a UV Stratalinker 1800 (1.2 x 10^5 microjoules/cm^2; Stratagene, USA).

Leaf material was pre-hybridised in 20 ml hybridisation buffer (see Table 2.1) at 65°C for 1 h and the buffer changed. Random primer-generated ^{32}P-labelled DNA (see section 2.2.11) using full-length TLCV DNA as template was added and the hybridisation done overnight at 65°C. Leaves were washed four times in 2 X SSC for 15 min, then once in 1 X SSC for 15 min and once in 0.5 X SSC for 15 min. Washes were done at 65°C. The leaves were placed on Whatman paper pre-wet with 2 X SSC, covered in plastic wrap and exposed to Biomax film (Kodak, USA) at -70°C in a film cassette containing a Biomax intensifying screen (Kodak, USA).

3.3 Results

3.3.1 GUS activity in TLCV promoter:GUS plants is abolished following TLCV infection

GUS activity in the TLCV promoter:GUS plants following TLCV infection was tested (see section 3.2.1).

Analysis of GUS activity in the uninoculated TLCV promoter:GUS plants showed the typical pattern of GUS expression for each transgenic line as described by Dry et al. (2000) (Fig. 3.1a). In contrast, GUS activity in the TLCV promoter:GUS plants inoculated with TLCV at 50 dpi could not be detected by histochemical assay (Fig. 3.1a). The abolition of GUS activity in inoculated plants by 50 dpi occurred in every plant in which systemic TLCV infection was established. GUS activity was not observed to recommence in plants silenced for GUS activity by TLCV infection. Analysis of GUS activity in tobacco inoculated with TLCV and carrying a CaMV 35S promoter:GUS transgene showed the same GUS activity at 50 dpi as an uninoculated CaMV 35S:GUS plant of the same age (Fig. 3.1a, bottom panels), indicating that the abolition of GUS activity following TLCV infection was limited to plants carrying TLCV-derived promoter sequences.

Analysis of the plants inoculated with TLCV by dot blot hybridisation showed that similar levels of TLCV DNA accumulated in the TLCV promoter:GUS plants and a control CaMV 35S:GUS plant (Fig. 3.1c). Following TLCV inoculation and the appearance of symptoms typical of TLCV infection at approximately 14 dpi, the progression of symptoms in the TLCV promoter:GUS plants was indistinguishable from those observed in infected CaMV 35S:GUS and non-transgenic plants. These observations together indicated that the replication and systemic spread of TLCV was not significantly affected in the presence of the TLCV promoter:GUS transgenes.

3.3.2 The silencing of GUS activity is initiated in vascular tissue and occurs in stem and preanthesis floral tissue

Plants containing the GUS gene driven by the TLCV V2 coat protein promoter (V2:GUSΔC, Fig. 1.3a) were selected for further experiments because of their high level of constitutive

GUS expression. The expression of GUS in V2:GUSΔC leaf, stem and preanthesis floral tissue from plants infected with TLCV was tested (see section 3.2.1).

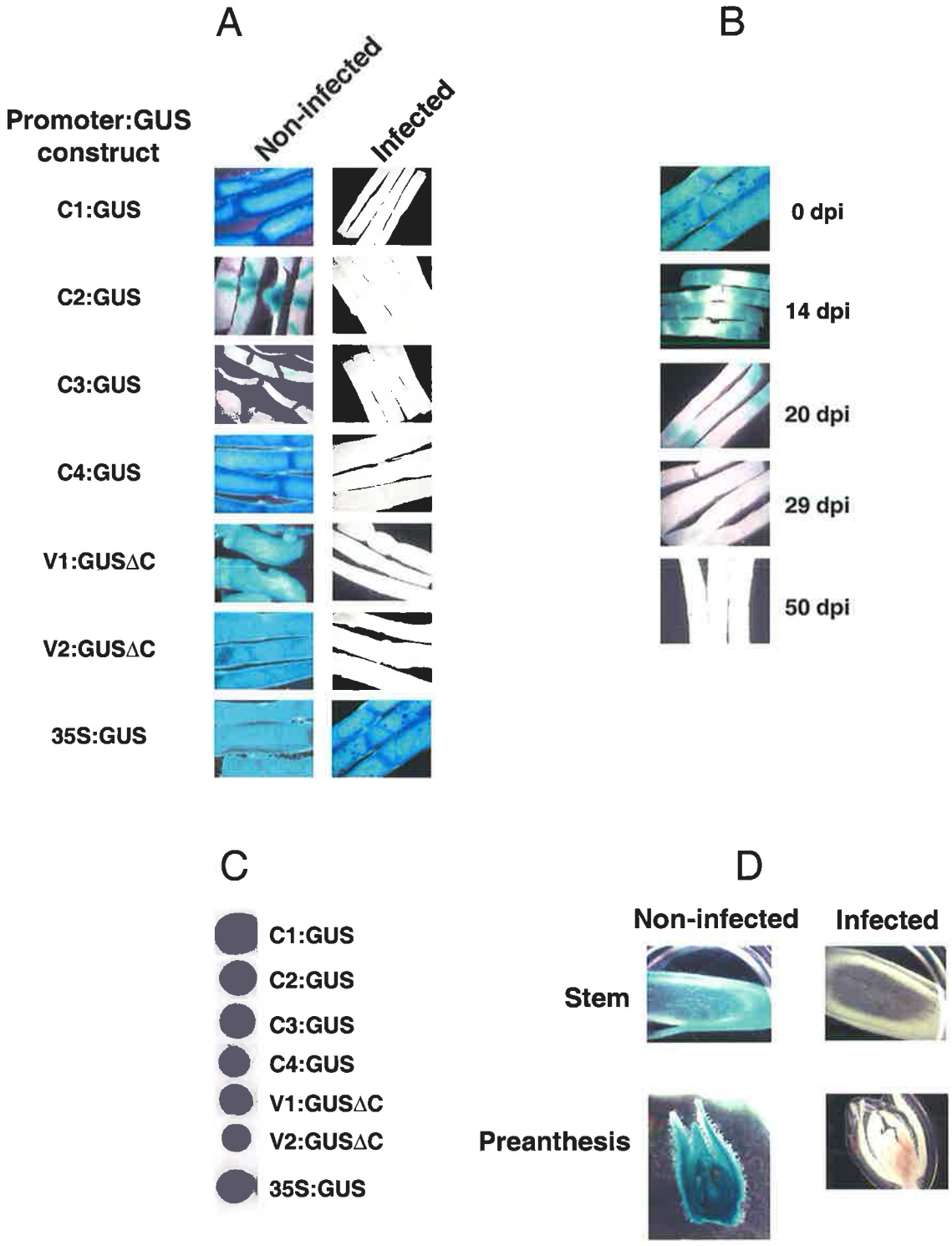


Figure 3.1 Evidence that TLCV infection silences GUS activity. (A), Sections of expanding leaves of TLCV promoter:GUS plants with and without TLCV infection were assayed for GUS activity by histochemical analysis. Sections from a CaMV 35S promoter:GUS plant are shown for comparison. Leaf sections were obtained at 50 days post inoculation (dpi). (B), Pattern of GUS activity in V2:GUSΔC leaf sections during the course of TLCV infection. (C), Accumulation of TLCV DNA in TLCV promoter:GUS plants as determined by dot blot hybridisation at 50 dpi. Dot blot of a CaMV 35S promoter:GUS plant infected with TLCV (50 dpi) is shown as a control. (D), Sections of V2:GUSΔC plants were assayed for GUS activity. Oblique slices of stem tissue were taken midway between internodes. Preanthesis floral tissue was bisected before analysis.

Analysis of GUS activity at 14 dpi showed that the silencing of GUS activity in the small expanding leaves of V2:GUSΔC plants was initiated in the vascular tissue collateral to the leaf mid-rib (Fig. 3.1b). Analysis at 20 and 29 dpi showed that silencing of GUS activity continued from the leaf vascular tissue into the surrounding mesophyll tissue towards the leaf margins, until GUS activity in the entire leaf was silenced at 50 dpi (Fig. 3.1b).

Analysis of GUS activity in the stem and preanthesis floral tissue of V2:GUSΔC plants showed constitutive GUS expression (Fig. 3.1d). In contrast, stem tissue from an infected V2:GUSΔC plant showed very weak to non-existent GUS activity (Fig. 3.1d). GUS activity could not be detected in dissected preanthesis floral tissue from an infected V2:GUSΔC plant. An attempt to analyse GUS activity in the root tissue of an uninoculated mature V2:GUSΔC plant was not successful.

3.3.3 Induction of silencing of GUS activity is limited to TLCV and a closely-related strain

The ability of viruses other than TLCV to induce the silencing of GUS activity in V2:GUSΔC plants was tested (see section 3.2.2).

Analysis of GUS activity in plants inoculated with either TLCV or TLCV-D1 showed the silencing of GUS activity at 50 dpi (Table 3.1). In contrast, plants inoculated with ACMV or TYLCSV showed similar levels of GUS activity at 50 dpi to the mock-inoculated plants (Table 3.1), which indicated that the silencing of GUS activity following virus infection was limited to TLCV or TLCV-D1. Dot blot hybridisation analysis of small expanding leaves in plants inoculated with ACMV and TYLCSV DNA confirmed that each virus had systemically infected the inoculated plants.

Thus, the silencing of GUS activity in V2:GUSΔC plants was limited to viruses with a high degree of sequence identity to the TLCV-derived promoter regions of the transgene.

Table 3.1 Silencing of GUS activity is virus-specific.[a]

Virus	% Identity[b]	GUS activity (dpi)		Virus infection
		0 days	50 days	
A. tumefaciens pBIN19	-	+	+	+
Tomato leaf curl virus	100	+	-	+
Tomato leaf curl virus-strain D1	84	+	-	+
African cassava mosaic virus	55	+	+	+
Tomato yellow leaf curl Sardinia virus	54	+	+	+

[a]Multiple V2:GUSΔC plants were infected by agroinoculation with the geminiviruses described and assayed for GUS activity by histochemical staining. Plants were assayed for systemic virus infection by dot blot hybridisation at 50 days post inoculation (dpi). Agroinoculation using *Agrobacterium* carrying an empty pBIN19 plasmid was included as a negative control.

[b]Percent nucleotide identity to the intergenic region and 5'-end of the C1 ORF of the wildtype TLCV sequence.

3.3.4. Silencing of GUS activity is independent of the TLCV C3, C4 and V1 gene products

V2:GUSΔC plants were inoculated with an infectious TLCV vector carrying a knock-out mutation in the C3 ORF (C3mut) to test the role of the C3 gene product in silencing (see section 3.2.3).

Analysis of GUS activity showed that activity was silenced in the six plants inoculated with the TLCV C3mut construct. Dot blot hybridisation analysis of the plants showed the accumulation of TLCV C3mut DNA, although to a lower level when compared to a control V2:GUSΔC plant inoculated with wild-type TLCV.

Previous experiments analysing the infection of V2:GUSΔC plants with TLCV C4 and V1 ORF knock-out constructs also showed the silencing of GUS activity by 50 dpi in systemically infected plants (I. Dry, unpublished result). Both the C4 and V1 ORF knock-out constructs are able to systemically infect tobacco.

Thus, the TLCV C3, C4 and V1 gene products were not required for the silencing of GUS activity in the V2:GUSΔC plants during systemic viral infection.

3.3.5 TLCV infection by graft transmission results in the silencing of GUS activity

The TLCV inoculation system used in the laboratory relies on the *A. tumefaciens*-mediated transfer of TLCV DNA from a binary vector to the adjacent plant cells at the site of inoculation. However it has been noted that *A. tumefaciens* is able to migrate in plant hosts from the initial site of infection, resulting in a systemic infection (Cubero et al. 1998). The role of *A. tumefaciens* in the silencing of GUS activity in V2:GUSΔC plants was tested. This

was done by graft-transmission of TLCV (from field-infected tomato) into V2:GUSΔC plants (see section 3.2.4).

Analysis of GUS activity in small expanding leaves of the V2:GUSΔC rootstocks showed that GUS activity was silenced. Dot blot hybridisation analysis of the rootstocks showed that they were systemically infected with TLCV. Thus, the use of an *Agrobacterium*-mediated inoculation system was not required for TLCV-induced GUS silencing.

3.3.6 Steady-state levels of GUS RNA in TLCV promoter:GUS plants are significantly reduced following TLCV infection

To determine whether the TLCV-induced silencing of GUS activity was due to interference with transcription or translation of the GUS gene, a comparison of steady-state GUS RNA levels in non-silenced and silenced C1:GUS and V2:GUSΔC plants was made by RT-PCR (see section 3.2.5).

RT-PCR products of approximately 500 bp were generated from RNA extracted from non-silenced C1:GUS and V2:GUSΔC plants using GUS-specific primers (Lane 2, Fig. 3.2a and Fig. 3.2c). These products were not due to contaminating DNA in the RNA samples as no products were detected in the absence of reverse transcriptase (Lanes 8-10, Fig. 3.2a and Fig. 3.2c). The identity of these products was shown to be from the GUS gene by Southern blotting (Fig. 3.2b and Fig. 3.2d).

No products were visible in reactions with GUS-specific primers using RNA from silenced C1:GUS and V2:GUSΔC plants (Lane 3, Fig. 3.2a and Fig. 3.2c), furthermore GUS DNA was not detected in these PCR reactions by Southern blotting (Fig. 3.2b and Fig. 3.2d). The difference in GUS RNA levels between non-infected and infected TLCV promoter:GUS could not be ascribed to different starting amounts of total RNA in the amplification reactions

as similar amounts of products were generated in Rubisco-specific PCR reactions (Lanes 5-7, Fig. 3.2a and Fig. 3.2c).

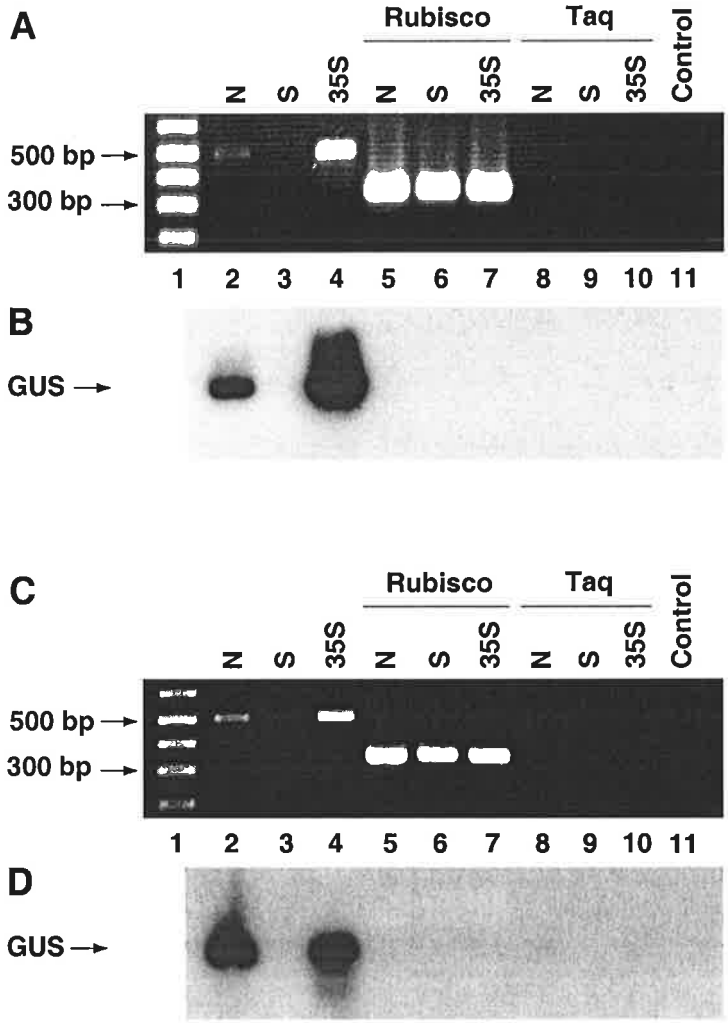


Fig. 3.2. Association of TLCV infection with a reduction in steady-state GUS RNA levels. GUS RNA was detected in total RNA extracts from C1:GUS and V2:GUSΔC plants by reverse-transcription PCR (RT-PCR) and the identity of reaction products confirmed by Southern hybridisation. (A), RT-PCR with RNA from C1:GUS plants. RT-PCR was done using GUS-specific (lanes 2-4, 8-10) or Rubisco-specific (lanes 5-7) primers. DNA contamination in RT-PCR reactions was checked by omission of reverse transcriptase (lanes 8-10). (B), Southern blot of RT-PCR products. Except for 35S, equal volumes of each RT-PCR reaction were blotted and probed with ^{32}P-labelled GUS DNA. The volume of 35S RT-PCR reaction used for blotting and probing was reduced by 75% to prevent the obscuring of adjacent signals on the film. (C), RT-PCR with RNA from V2:GUSΔC plants. (D), Southern blot of RT-PCR products. N: non-silenced; S: silenced. 35S: Cauliflower mosaic virus 35S promoter:GUS plants infected with TLCV; Control: water.

These results indicated that TLCV-induced silencing of GUS activity occurred at the transcriptional or post-transcriptional level.

3.3.7 Transcription of GUS in V2:GUSΔC plants is abolished following TLCV infection

To determine whether TLCV-induced silencing of GUS activity occurred at the transcriptional or post-transcriptional level, nuclear run-on analysis of GUS transcription was done on V2:GUSΔC plants (see section 3.2.6).

Run-on transcripts purified from non-silenced V2:GUSΔC nuclei hybridised to both GUS and Rubisco (Fig. 3.3). In contrast, run-on transcripts from silenced V2:GUSΔC nuclei hybridised only to Rubisco DNA, indicating that GUS transcripts were not synthesised in silenced V2:GUSΔC leaf tissue (Fig. 3.3). The absence of GUS transcripts from silenced V2:GUSΔC nuclei was not due to a reduction in total RNA added to the hybridisation, as more Rubisco transcripts were detected in silenced nuclei than in non-silenced nuclei.

Thus, the TLCV-induced silencing of GUS activity was due to the abolition of transcription from the V2:GUSΔC transgene.

3.3.8 Wholemount *in situ* hybridisation to detect TLCV DNA in intact V2:GUSΔC leaf tissue

Species within the genus *Begomovirus* exhibit different tissue tropisms. During infection of *N. benthamiana*, BGMV is limited to invasion of vascular cells. In contrast TGMV invades both the vascular and mesophyll cells of *N. benthamiana* (Morra and Petty 2000). TLCV-induced silencing of GUS expression occurred in both the vascular and mesophyll leaf tissue of the TLCV promoter:GUS plants (Fig. 3.1b). Therefore the question arose as to whether TLCV DNA from the invading virus was present in both the vascular and mesophyll tissue in leaves

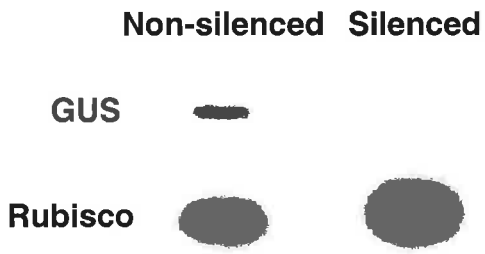


Figure 3.3. Association of TLCV infection with a lack of GUS transcription. Nuclear run-on analysis of nuclei isolated from non-silenced or silenced V2:GUSΔC leaves was done at 50 days post infection. ^{32}P-labelled total RNA purified from 3.5 x 10^6 nuclei was used to probe a slot blot containing 5 μg of full-length GUS and 0.5 μg of a 300 bp fragment of Rubisco DNA.

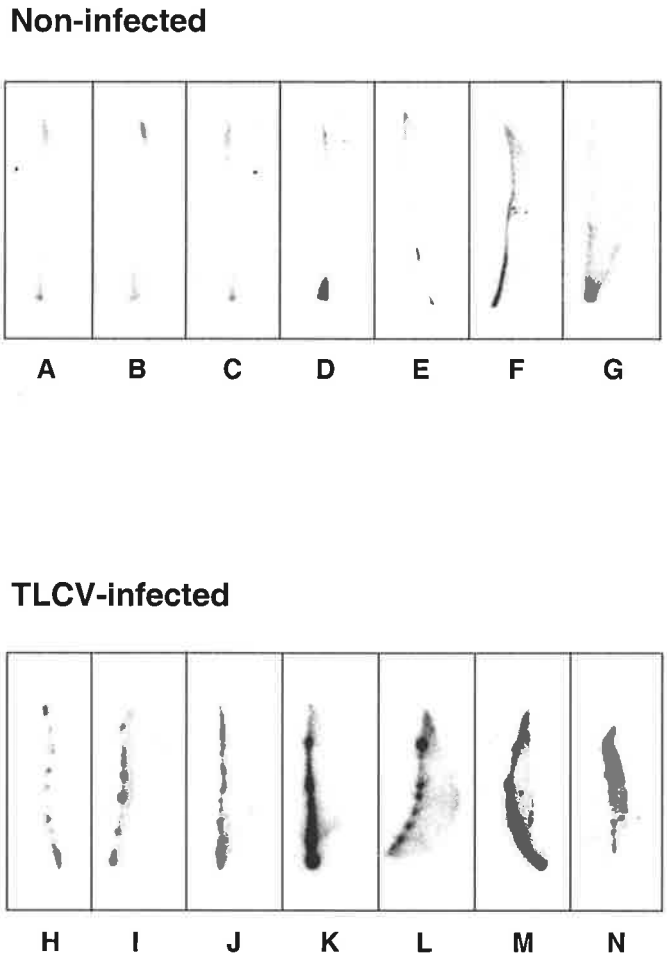


Figure 3.4 Wholemount *in situ* hybridisation of V2:GUSΔC leaf tissue for replicating TLCV DNA. Dissected leaf tissues from plants were prepared for wholemount *in situ* hybridisation and hybridised to ^{32}P-labelled TLCV DNA generated from a full-length TLCV DNA template. (A-G), leaf tissues from non-infected plants. (H-N), leaf tissues from TLCV-infected plants. Leaf pieces were either the intact mid-rib associated tissue from a whole leaf (A-D, H, I) or the intact mid-rib associated tissue plus the mesophyll tissue from one side of the leaf (E-G, J-N).

silenced for GUS expression. To address this question, a procedure was used to detect TLCV DNA in intact leaf tissue. This was done by hybridising a TLCV DNA probe with V2:GUSΔC leaf pieces (see section 3.2.7).

Leaf pieces derived from non-infected V2:GUSΔC plants exhibited non-specific hybridisation signals that were limited to the periphery of the leaf pieces (Fig. 3.4f and 3.4g) or to the ends of the mid-rib associated tissue (Fig. 3.4a-e). Leaf pieces from TLCV-infected V2:GUSΔC plants also exhibited the pattern of non-specific hybridisation. However a second pattern of hybridisation to discrete loci was also observed to occur in the infected samples (Fig. 3.4h-n). This pattern was not seen in the non-infected leaf pieces and was clearly distinguishable from the non-specific background observed in all leaf pieces. The discrete hybridisation loci were generally limited to the mid-rib associated tissue of the infected samples (Fig. 3.4h-m) but were also observed to occur in both mid-rib associated and mesophyll tissue (Fig. 3.4n). The pattern of discrete hybridisation loci suggested that replicating TLCV DNA was being detected and that it was more highly concentrated in the vascular tissue associated with the leaf mid-rib.

3.4 Discussion

To resolve whether TLCV gene products were able to alter transcription directed from integrated TLCV promoter sequences, plants from six TLCV promoter:GUS transgenic lines (Dry et al. 2000) were inoculated with TLCV. Following systemic virus infection, expression directed by the integrated TLCV promoters could not be detected in the leaf tissue of the plants, indicating the virus-induced silencing of the TLCV promoter:GUS transgenes.

Silencing was both sequence- and virus-specific. TLCV–induced silencing was limited to transgenes carrying TLCV-, but not CaMV-derived promoter sequences (Fig. 3.1a). Silencing of the V2:GUSΔC transgene was induced exclusively by infection with TLCV, or the closely

related D1 strain (Table 3.1). Expression of the TLCV C3, C4 and V1 gene products during TLCV infection were not required for silencing of the V2:GUSGΔC transgene (see section 3.3.4), indicating that silencing was not mediated by these virus proteins.

Silencing of transgene expression in the leaves of V2:GUSΔC plants was observed initially in the vascular tissue and to subsequently occur in the mesophyll tissue (Fig. 3.1b). Wholemount *in situ* hybridisation to detect TLCV DNA in the leaves of silenced V2:GUSΔC plants suggested that virus spread was limited to vascular-associated tissue (Fig. 3.4). This preliminary experiment was in agreement with recent *in situ* hybridisations to detect TLCV DNA in the leaf tissue of host species. Analysis in the laboratory of Dr Ali Rezaian (CSIRO Plant Industry, South Australia) of leaf cross-sections has revealed the presence of TLCV exclusively in the phloem cells of infected tomato and *N. benthamiana* (S. Rasheed, manuscript in preparation). It should be noted that these results do not rule out a low level of TLCV DNA in mesophyll cells. Taken together, the hybridisation results suggested that silencing of the TLCV promoter:GUS transgenes occurred in both infected and non-infected host cells. TLCV-induced silencing was restricted to transgenes carrying TLCV-derived sequences, suggesting a requirement for sequence homology between the virus and the transgene. One explanation for the spread of transgene silencing from infected to non-infected cells would be the movement of a mobile silencing signal which contains a nucleic acid component to mediate sequence-specific silencing.

Silencing of transgene expression occurred in the preanthesis floral tissue of infected V2:GUSΔC plants (Fig. 3.1d). The bipartite geminivirus *Bean dwarf mosaic virus* has been observed to infect the flower, pod and seed coat tissue of *P. vulgaris* (Sudarshana et al. 1998). Thus, silencing of V2:GUSΔC transgene in floral tissue may have been mediated by TLCV infection in the floral cells. However, the ability of TLCV to colonize cells in flower tissue has not as yet been tested.

The inactivation of the integrated TLCV promoters in transgenic tobacco plants following TLCV infection represented a new example of VITGS. To date, this is the first report of VITGS associated with a geminivirus infection.

Chapter 4

Sequence-specific DNA hypermethylation following TLCV-mediated VITGS

4.1 Introduction

Methylation of cytosine increases the information content of DNA. The total methylcytosine content of plant DNA ranges from 6 % in Arabidopsis (*Arabidopsis thaliana*) to 33 % in rye (Thomas and Sherratt 1956) with methylcytosine residues occurring in any sequence context. The most common location of methylcytosines is in sequences that are identical when read from 5' to 3' on each strand. These symmetric motifs comprise the CpG and CpNpG sequences (where N is any base but G). Methylation patterns of symmetric motifs are transmitted through rounds of DNA replication by the maintenance methyltransferases. These enzymes, which have a preference for hemi-methylated DNA and act after DNA synthesis, modify the unmethylated symmetric cytosines in the newly synthesised DNA strand (Bird and Southern 1978). Thus, the strand symmetry of CpG and CpNpG motifs provide a mechanism for the clonal transmission of methylation patterns. The methylation of cytosines in asymmetric (CpNpN) motifs indicates the existence of *de novo* methyltransferase enzymes in plants (Finnegan and Kovac 2000), which are presumably capable of methylating cytosine residues in any sequence motif.

The majority of techniques for analysing cytosine methylation of DNA take advantage of the sensitivity of a number of restriction enzymes to cytosine methylation of their cognate DNA recognition sequence. Digestion reactions using a combination of methylation-sensitive and –insensitive isoschizomer pairs allow the analysis of methylcytosines occurring in specific DNA sequences.

An alternative to restriction enzyme digestion is the analysis of cytosine methylation by bisulfite modification and sequencing. This method takes advantage of the resistance of methylcytosine residues in ssDNA to modification by bisulfite, while unmethylated cytosine

in ssDNA is converted to uracil. The treated DNA can then be amplified by PCR, cloned and sequenced. Following bisulfite modification, the strands of treated DNA are no longer complementary. Thus, the amplification of target DNA by PCR is strand-specific.

Oligonucleotide primers for the analysis of bisulfite-treated DNA can be designed to specifically bind to DNA which is unmethylated at symmetric cytosines, thus biasing the PCR towards the amplification of sequences that have a low level of cytosine methylation (Thomassin et al. 1999). Alternatively, the primers can be designed to amplify sequences which have a higher level of cytosine methylation.

Transcriptional gene silencing induced by the RNA viruses PVX and TRV was correlated with cytosine hypermethylation of the virus-derived genomic sequences (Jones et al. 1999; Jones et al. 2001). Both symmetric and asymmetric cytosines became hypermethylated following TRV-induced silencing. In progeny that inherited the silenced phenotype, only hypermethylation of symmetric cytosines was retained. In contrast to the RNA viruses, transcriptional gene silencing induced by the DNA virus CaMV was not associated with hypermethylation of CaMV-derived genomic sequences (Al-Kaff et al. 2000).

The aim of the work in this chapter was to analyse cytosine methylation in the V2:GUSΔC transgene following TLCV-induced silencing. Analysis of methylation by restriction enzyme digestion could not be done because TLCV dsDNA contaminated DNA samples from silenced V2:GUSΔC promoter:GUS plants. Therefore, analysis was done by bisulfite modification and sequencing of the V2:GUSΔC DNA. Extensive hypermethylation of symmetric and asymmetric cytosines in the TLCV-derived sequences of the V2:GUSΔC transgene in silenced plants was detected. In contrast, cytosines in the 5'-end of the GUS ORF in silenced plants were hypomethylated.

4.2 Materials and methods

4.2.1 Bisulfite modification and sequencing of tobacco genomic DNA

Tissue from either five non-silenced or five silenced V2:GUSΔC plants was pooled and total DNA extracted (see section 2.2.12).

Bisulfite modification and sequencing was done according to Wang and co-workers (2001) with the following modifications.

Two pairs of primers, each of which amplified the same region of the virion-sense strand of the V2:GUSΔC transgene, were designed (see Fig. 4.1a). One pair was designed to bind to bisulfite-treated DNA unmethylated at CpG dinucleotides. The second pair was designed to bind to bisulfite-modified DNA methylated at CpG dinucleotides. Forward and reverse primers from either primer pair were used in various combinations. One primer combination was successfully used to amplify a product from bisulfite-modified DNA from V2:GUSΔC tissue, consisting of a forward primer to bind to DNA methylated at CpG dinucleotides and a reverse primer to bind to DNA unmethylated at CpG dinucleotides. The primers used were TLCVfor and GUSrev (see Table 2.2). Primer pairs also used for amplification from the bisulfite-treated DNA were: TLCV39-68 and GUS127-157 (for amplification of the unmodified V2:GUSΔC transgene, see Fig. 4.1a for location of primers); 5Sfor and 5Srev (for amplification of bisulfite modified tobacco 5S rDNA sequences) (Table 2.2).

PCR products were purified using a QIAGEN PCR Purification kit, ligated into the vector pGEM-T-Easy (see section 2.2.5) and transformed into *E. coli* XL1-Blue cells (see section 2.2.6). Plasmid DNA was prepared from *E. coli* using a QIAGEN Spin Miniprep kit and sequenced with T7 or SP6 primers (see section 2.2.10).

PCR amplification was carried out on the top strand of V2:GUSΔC DNA using a GUS-specific reverse primer to avoid amplification from genomic TLCV DNA present in silenced plants.

4.3 Results

4.3.1 Amplification from the promoter:GUS ORF junction of the genomic copy of V2:GUSΔC transgene following bisulfite modification

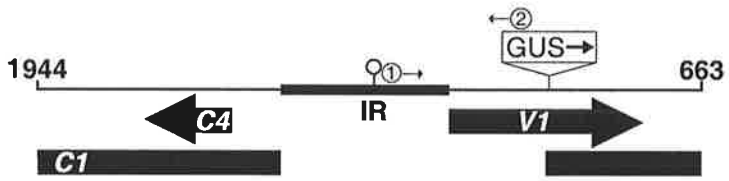
4.3.1.1 DNA amplification of bisulfite-modified V2:GUSΔC transgene DNA

PCR amplification of DNA from both non-silenced and silenced tissue was achieved using a forward primer to bind "methylated" DNA and a reverse primer to bind "unmethylated" DNA (Lanes 9 and 10, Fig 4.1b). The amplified PCR product corresponded to the junction between the 3'-end of the TLCV-derived promoter sequence and the 5'-end of the GUS ORF sequence (see Fig 4.1a for location of primers). The PCR products were 430 bp in length, including 275 bp of promoter sequence and 155 bp of GUS ORF sequence.

4.3.1.2 DNA amplification to test for proper bisulfite modification of the genomic DNA

PCR amplification from the bisulfite-modified DNA was done using two other pairs of primers. Amplification was done with a primer pair designed to bind specifically to unmodified DNA and amplify the same region of the V2:GUSΔC transgene as described in Figure 4.1a (Lanes 3 and 4, Fig. 4.1b). These reactions did not result in a detectable product, which indicated that unmodified DNA was not present in the bisulfite-treated V2:GUSΔC samples.

A



B

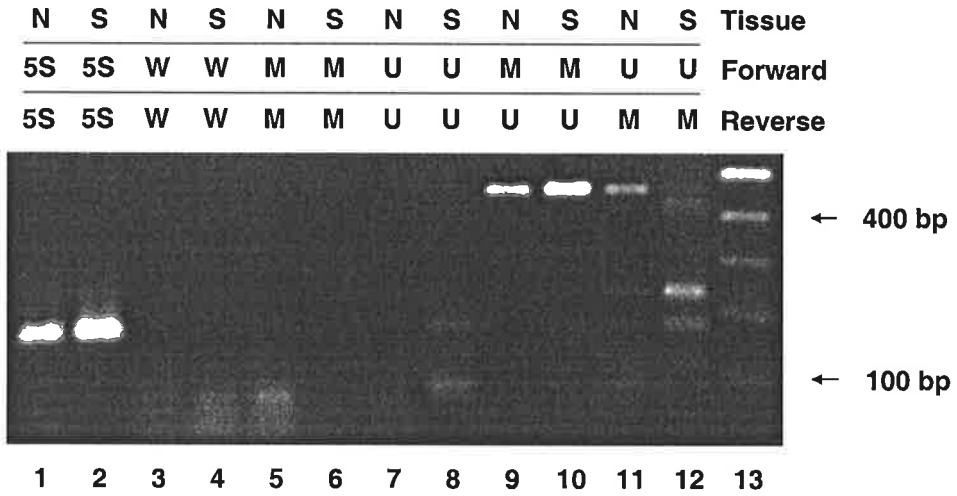


Figure 4.1 Amplification of DNA from the V2:GUSΔC transgene following bisulfite modification. (A), Schematic representation of the V2:GUSΔC construct. See Figure 1.3 for description. Circled numbers (1 and 2) and small arrows above the construct denote the position of the oligonucleotide primers used for amplification of the bisulfite-modified V2:GUSΔC transgene. TLCV forward primer (1, TLCV nucleotide positions 39-68); GUS reverse primer (2, GUS nucleotide positions 127-143). (B), PCR of bisulfite-modified genomic DNA from non-silenced and silenced V2:GUSΔC leaf tissue. DNA size markers (lane 13; Invitrogen 1 kb+ DNA Ladder). N: bisulfite-modified genomic DNA from non-silenced V2:GUSΔC tissue; S: bisulfite-modified genomic DNA from silenced V2:GUSΔC tissue; Tissue: V2:GUSΔC leaf tissue used for genomic DNA extraction; 5S: oligonucleotide primers for amplification of bisulfite-modified 5S rDNA tobacco DNA; W: oligonucleotide primer for amplification of unmodified V2:GUSΔC DNA; M: oligonucleotide primer for amplification of bisulfite-modified V2:GUSΔC DNA methylated at CpG motifs; U: oligonucleotide primer for amplification of bisulfite-modified V2:GUSΔC DNA not methylated at CpG motifs; Forward: forward oligonucleotide primer; Reverse: reverse oligonucleotide primer.

Amplification was also done using a primer pair to bind to the top strand of tobacco 5S rDNA sequences modified by bisulfite (Lanes 1 and 2, Fig. 4.1b). The 5S primers have been described previously and direct amplification of an approximately 190 bp product, which includes 88 bp of 5S rDNA sequence (Fulnecek et al. 1998). Products of approximately 190 bp were detected in both reactions using the 5S primers. The PCR products generated using the 5S primers were cloned and one clone from either non-silenced or silenced tissue was sequenced. The cytosine methylation rates in DNA from non-silenced tissue (48 %) and silenced tissue (52 %) were comparable to the published average methylation rate of 53 % for the top strand of the tobacco 5S rDNA sequence (Fulnecek et al. 1998). Taken together, these results indicated that the bisulfite modification of the genomic V2:GUSΔC DNA was complete.

4.3.2 Silencing of GUS expression is correlated with cytosine hypermethylation of the TLCV-derived sequences of the V2:GUSΔC transgene

The products of the amplification of the V2:GUSΔC transgene (Lanes 9 and 10, Fig 4.1b) were cloned and six individual clones derived from either non-silenced or silenced tissue were sequenced (see section 4.2.1). Figure 4.2 represents the sequences of the clones.

4.3.2.1 The TLCV-derived sequences of the V2:GUSΔC transgene in silenced tissue are hypermethylated

Cytosine methylation analysis using bisulfite modification and sequencing revealed significant hypermethylation of the sense strand of the V2:GUSΔC promoter sequence in silenced tissue (Fig. 4.2, compare cytosine methylation between "N" and "S"). The average cytosine methylation rate (i.e. the percentage of all cytosines which are methylated) in the

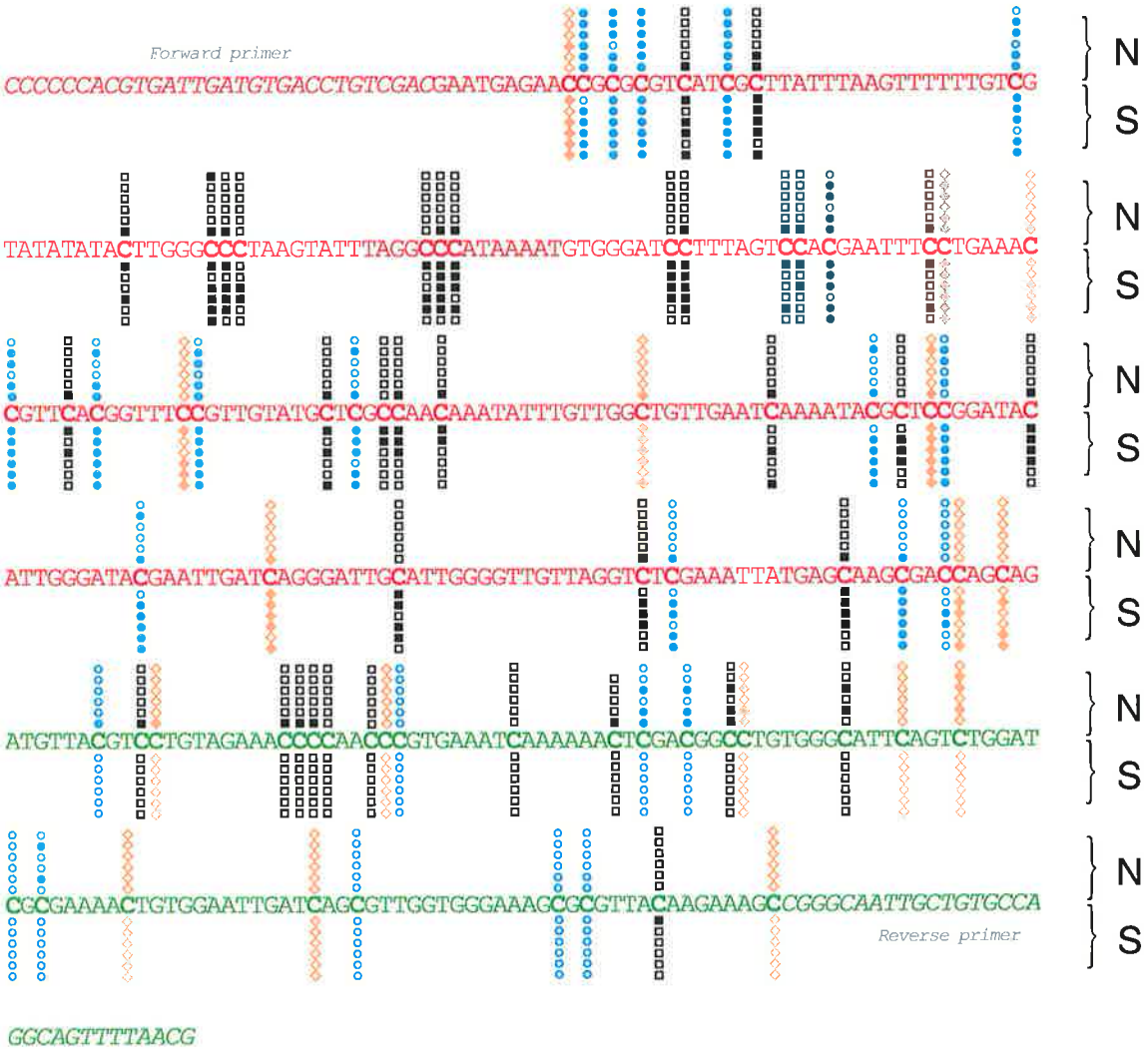


Figure 4.2 Cytosine methylation in the top strand of the V2:GUSΔC transgene. Bisulfite modification and sequencing of genomic V2:GUSΔC DNA was done and a 430 bp product amplifed from the V2:GUSΔC transgene. The PCR product was cloned and individual (six) clones sequenced. The transgene sequence of the PCR product is shown. TLCV-derived promoter sequences are in red, GUS ORF sequences are in green. The primer binding sequences are italicised. Cytosines potentially subject to bisulfite modification and sequencing are in bold. Symbols above and below the transgene sequence indicate cytosines analysed for methylation status and mapped to this position. Symbols are coded indicating the cytosine sequence motif: cytosines in CpG motifs are blue circles; cytosines in CpNpG motifs are orange diamonds; and cytosines in asymmetric (CpNpN) motifs are black squares. Open symbols represent an unmethylated cytosine; closed symbols represent a methylated cytosine. N: cytosine methylation in transgene DNA from non-silenced tissue; S: cytosine methylation in transgene DNA from silenced tissue.

TLCV-derived promoter sequences was 30 % for non-silenced tissue and 69 % for silenced tissue.

4.3.2.2 The distribution of cytosine methylation by sequence motif is altered following silencing of the V2:GUSΔC transgene

Cytosines can be classified into symmetric (CpG and CpNpG) or asymmetric (CpNpN) motifs. In non-silenced tissue, the majority of methylated cytosines were in CpG motifs (Fig. 4.3a). In silenced tissue, the majority of methylated cytosines (59 %) were in CpNpG and asymmetric cytosines (Fig. 4.3b). Thus, TLCV infection and transgene silencing correlated with a change in the distribution of methylation in the TLCV-derived V2:GUSΔC sequences, from methylation predominantly of CpG motifs to more evenly distributed methylation in both symmetric and asymmetric motifs.

4.3.2.3 The proportion of cytosines in each motif classification which are methylated increases following silencing of the V2:GUSΔC transgene

An alternative approach to analysing cytosine methylation is to determine the proportion of cytosines in each motif which are methylated. The proportion of methylated cytosines in each motif increased following transgene silencing (Table 4.1). In silenced tissue, almost all (91 %) of CpG motifs were methylated. Following silencing, methylation of CpNpG and asymmetric motifs increased by approximately 4.7-fold and 3.8-fold, respectively.

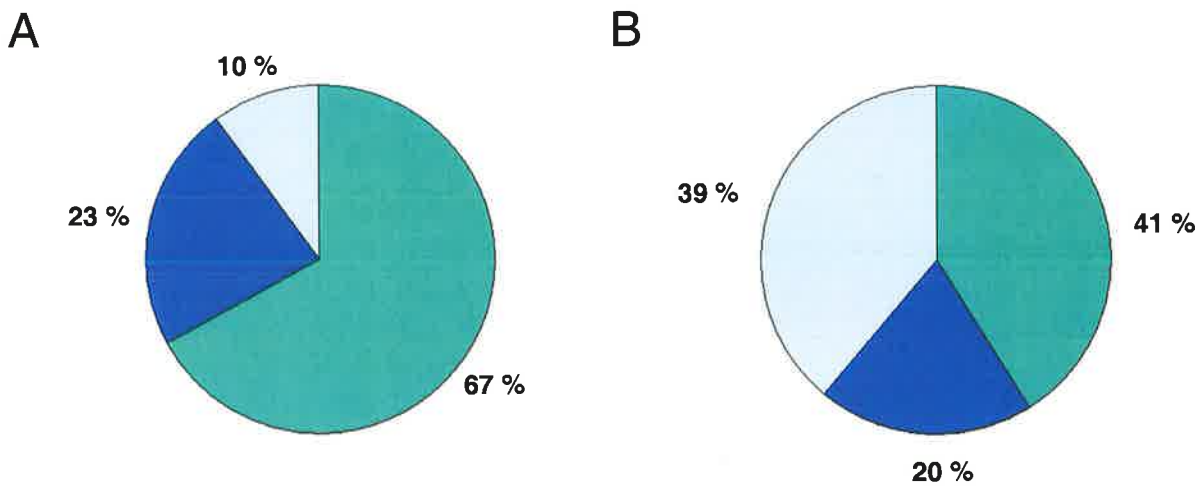


Figure 4.3 The distribution of cytosine methylation by sequence motif in the V2:GUSΔC transgene. The distribution was calculated from the results of bisulfite modification and sequencing the V2:GUSΔC transgene. The proportion of CpG, CpNpG and CpNpN methylation is represented by the green, blue and purple sections of the graph, respectively. A, cytosine methylation in the non-silenced V2:GUSΔC transgene. B, cytosine methylation in the silenced V2:GUSΔC transgene.

Table 4.1 The proportion of cytosines methylated in the V2:GUSΔC transgene in each motif classification.[a]

Sequence motif	Non-silenced	Silenced
CpG	68	91
CpNpG	17	80
CpNpN	14	53

[a]The percentage of cytosines which are methylated in each sequence motif was calculated for the TLCV-derived sequences of the V2:GUSΔC transgene analysed by bisulfite modification and sequencing.

4.3.2.4 Hypermethylation occurs in most leaf cells of the silenced V2:GUSΔC plants

In Figure 4.2 the ordering of symbols above and below each cytosine residue is consistent for each cytosine residue, allowing the distribution of methylated cytosines in each clone to be mapped. The clones generated from bisulfite-modified DNA from both non-silenced and silenced plants displayed varying degrees of methylation in the TLCV-derived transgene sequences. Rates of methylation in clones from non-silenced tissue ranged from 8 – 37 %, while rates in clones from silenced tissue ranged from 58 - 80 %. This suggested that hypermethylation of the V2:GUSΔC transgene occurred in most, if not all, leaf cells from silenced tissue.

4.3.3 The GUS-derived sequences of the V2:GUSΔC transgene in silenced tissue are hypomethylated

Methylation analysis of the 5'-end of the GUS ORF from the V2:GUSΔC transgene revealed a small but significant decrease in cytosine methylation following silencing (Fig. 4.2). The average methylation rate in non-silenced tissue was 17 % and decreased to 0.6 % in silenced tissue. Methylation of only one cytosine (in an asymmetric motif) was detected in six clones derived from silenced tissue, indicating the near lack of methylation in the GUS sequences screened. Thus, the TLCV-induced silencing of expression from the V2:GUSΔC transgene resulted in hypermethylation of the homologous TLCV-derived promoter sequences and hypomethylation of the non-homologous GUS ORF sequences of the transgene.

4.4 Discussion

VITGS induced by RNA viruses, but not DNA viruses has been found to be associated with cytosine hypermethylation of host genomic sequences. Hypermethylation associated with PVX (Jones et al. 1999) or TRV (Jones et al. 2001) infection was limited to genomic

sequences homologous to sequences carried by either virus. Thus, hypermethylation of the integrated virus-derived promoters, but not the downstream ORF sequences, was observed. This indicated that VITGS was associated with sequence-specific hypermethylation. TLCV-induced silencing of the V2:GUSΔC transgene was associated with hypermethylation of the TLCV-derived, but not the GUS ORF, transgene sequences. Thus, VITGS associated with both RNA and DNA virus infections results in the sequence-specific hypermethylation of homologous genomic sequences.

Hypermethylation of both symmetric and asymmetric cytosines in the TLCV-derived sequences of the V2:GUSΔC transgene occurred following TLCV infection (Fig. 4.2 and Table 4.1). This suggested that methylation was mediated by recruitment of host *de novo* methylases to the V2:GUSΔC transgene sequence. Similar to VITGS associated with PVX and TRV, not every cytosine in the virus-derived genomic sequences was fully methylated (Jones et al. 1999; Jones et al. 2001) (Table 4.1). Thus, the silencing of genomic promoter sequences by VITGS does not require or result in the complete methylation of promoter cytosine residues.

Currently there is limited information available on bisulfite sequencing of host DNA methylation following plant pathogen-homologous transgene interactions. Analysis of an integrated satellite of the *Cereal yellow dwarf virus* (CYDVsat) in tobacco showed that extrachromosomal replication of the homologous CYDVsat RNA led to transgene hypermethylation, which was mostly restricted to CYDVsat-derived sequences (Wang et al. 2001). Following CYDVsat infection, cytosine methylation was observed in 85 % of symmetric motifs and 57 % of asymmetric motifs, a pattern closely mirrored in TLCV-induced hypermethylation. Outwardly, this suggested that the *de novo* methylation following TLCV and CYDVsat infection was biased towards the methylation of symmetric cytosines. In the V2:GUSΔC transgene sequence following silencing, methylation of symmetric cytosines

occurred approximately 1.7X more frequently than methylation of asymmetric cytosines. However, there was approximately 1.7X more asymmetric cytosines available to be methylated in the non-silenced transgene sequence than available symmetric cytosines. Random *de novo* methylation of the transgene sequence would likely have resulted in the methylation of a smaller proportion of the more common asymmetric cytosines, and the methylation of a higher proportion of the less common symmetric cytosines. Therefore random *de novo* methylation could account for the observation that a higher percentage of symmetric cytosines than asymmetric cytosines were methylated following TLCV infection. Taken together, this suggested that methylation of the V2:GUSΔC transgene sequence probably occurred in a random manner.

As discussed in Chapter 3, silencing of GUS activity appeared to occur in both non-infected and TLCV-infected cells of the V2:GUSΔC plants. This is supported by the detection of hypermethylation of the V2:GUSΔC transgene sequences of most, if not all, leaf cells (Fig. 4.2). As an explanation for the occurrence of silencing in both non-infected and infected host cells, a mobile silencing signal with a nucleic acid component to direct the silencing of TLCV-homologous genomic sequences was proposed (see section 3.4). A further proposal is that the silencing signal was also responsible for the sequence-specific hypermethylation of the V2:GUSΔC transgene. In this scenario, the nucleic acid component of the silencing signal would be homologous only to the TLCV-derived sequences of the V2:GUSΔC transgene.

Chapter 5

Inheritance of TLCV-mediated VITGS

5.1 Introduction

Infection of 35S:GFP *N. benthamiana* plants with recombinant TRV carrying a CaMV 35S insert resulted in transcriptional silencing of the transgene (Jones et al. 2001). Furthermore, progeny derived from TRV-silenced 35S:GFP plants also displayed a silenced phenotype, demonstrating the heritability of VITGS. Maintenance of the silenced phenotype in the progeny was dependent on the activity of the cytosine methyltransferase METI. The progeny from the silenced 35S:GFP parent initially displayed a silenced phenotype, however with further growth transgene expression was restored in 70 % of progeny.

The silenced state of a transgene due to VITGS can be transmitted through meiosis to progeny. This, along with the restoration of transgene expression in progeny, indicates that VITGS can result in the epimutation of genomic sequences. Central to mechanisms of epigenetic control are the changes in DNA accessibility that are brought about by alterations in chromatin structure. Chromatin is the dynamic polymer of genomic DNA that is highly folded, constrained and compacted by histone and non-histone proteins. Higher order chromatin structures have been associated with transcriptional "on" and "off" states, which can be propagated through mitosis and meiosis (Jenuwein and Allis 2001). Euchromatic domains of genomic DNA are "open" and accessible to transcriptional machinery, and are generally associated with active transcription of ORFs within the domain. Heterochromatic domains are "closed" and inaccessible to transcriptional machinery, and are generally associated with a lack of transcription. Furthermore, heterochromatin is associated with the formation of higher-order multimeric protein structures which are resistant to nuclease attack (Grewal and Elgin 2002).

The qualities of higher-order chromatin structures (euchromatin and heterochromatin) have been proposed to be largely dependent on the local concentration and combination of differently modified histone proteins (Jenuwein and Allis 2001). As a general guide, heterochromatin is associated with histone hypophosphorylation and hypoacetylation. Additionally, histone methylation and the recruitment of the histone chromosomal 1 (HP1) protein is strongly linked to heterochromatic states (Lachner et al. 2001).

The formation of heterochromatic domains of DNA is often linked to cytosine hypermethylation (Felsenfeld and Groudine 2003). Recently, biochemical pathways between hypermethylation and the formation of heterochromatin have been identified. Sequences of mammalian hypermethylated DNA attract the methyl-CpG-binding protein MeCP2, which in turn induces the formation of a local heterochromatic state (Nan et al. 1998). A link between histone methylation, HP1 recruitment and CpNpG methylation has been identified in Arabidopsis (Jackson et al. 2002).

To determine whether VITGS due to TLCV infection resulted in epimutation of the TLCV promoter:GUS transgenes, GUS activity was analysed by histochemical and quantitative assays in progeny derived from silenced plants. This chapter reports that transgene expression in progeny from silenced C1:GUS and C4:GUS plants was down-regulated. Transgene expression in progeny from silenced V1:GUSΔC and V2:GUSΔC plants was initially silenced, however restoration of expression was observed in mature V2:GUSΔC progeny. Furthermore, the maintenance of the down-regulated or silenced phenotypes in progeny was disrupted by treatment with modifiers of host epigenetic regulation.

5.2 Materials and methods

5.2.1 Analysis of transgene activity in progeny TLCV promoter:GUS plants

5.2.1.1 Analysis of progeny TLCV promoter:GUS plants

T_1 TLCV promoter:GUS plants were inoculated with TLCV (see section 2.2.20). Plants were tested at 50 dpi for GUS activity (see section 2.2.21) and virus infection (see section 2.2.18). The T_1 plants were allowed to self-fertilise and the T_2 seeds collected. The T_2 seeds were germinated *in vitro* on solid media containing the antibiotic kanamycin (see section 5.2.2) to select for the germination of transgenic plants. The GUS activity in the leaf tissue of the T_2 plants was tested by histochemical assay (see section 2.2.21). The assays were done either on *in vitro* plants at 14 days post germination (dpg) or after the plants had been re-planted in soil and maintained in a glasshouse (43 dpg).

Dot blot hybridisation assays for TLCV infection were done on progeny derived from infected TLCV promoter:GUS plants (see section 2.2.18). The V1:GUSΔC and V2:GUSΔC T_2 progeny were assayed for TLCV infection. As a positive control for the dot-blot hybridisation assay, a tomato plant inoculated with TLCV was also tested. V2:GUSΔC progeny were tested for TLCV infection at either 45 dpg (4 plants) or 140 dpg (2 plants). V1:GUSΔC progeny (7 plants) were tested at 48 dpg.

5.2.1.2 Extended analysis of progeny TLCV promoter:GUS plants

T_1 plants (two) from each TLCV promoter:GUS line were inoculated with TLCV (see section 2.2.20). The plants were shown to be silenced for GUS activity (see section 2.2.20) and positive for TLCV infection (see section 2.2.18) at 50 dpi. The plants were allowed to self-

fertilise and the T_2 seeds collected. As controls, T_2 seeds were also collected from two non-inoculated plants for each TLCV promoter:GUS line.

The T_2 seeds were germinated *in vitro* on kanamycin (see section 5.2.2) to select for transgenic plants. After two weeks the progeny plants were re-planted in soil and maintained in a glasshouse.

The plants were inoculated at 75 dpg with TLCV (see section 2.2.21). Analysis of GUS activity (see section in 2.2.20) in the T_2 plants was done at 10, 75 and 125 dpg (50 days post inoculation). The plants were tested for TLCV infection (see section 2.2.18) at 50 days post inoculation (125 dpg). The progeny of the C2:GUS transgenic line could not be analysed as the seed collected from infected C2:GUS plants was not viable.

5.2.2 In vitro germination of tobacco seeds

Tobacco seeds were soaked in 4 % (v/v) hypochlorite for 30 min and then washed in sterile water for five times for 2 min each wash. Seeds were germinated on Murashige and Skoog agar (Horsch et al. 1985) containing 3 % (w/v) sucrose and 50 μg ml^{-1} kanamycin at 23°C under artificial light (150 μmoles m^{-2}) for a 16 h photoperiod. As required, 75 μg ml^{-1} 5-azacytidine (Sigma), 10 μM trichostatin A (Sigma) or 10 mM sodium butyrate (Sigma) were added to medium.

5.2.3 Quantitative analysis of GUS activity

Analysis of GUS activity was done on *in vitro*-germinated seedlings at 10 dpg (see section 5.2.2). Leaf and stem tissue of seedlings was harvested, pooled and weighed. The tissue was ground in liquid nitrogen, resuspended in 2.5 vol. extraction buffer (EB: 50 mM sodium phosphate pH 7, 1 mM EDTA, 0.1 % (v/v) sarcosyl, 0.1 % (v/v) Triton X-100, 0.078 % (v/v) β-mercaptoethanol) and vortexed for 15 s before centrifugation at 4°C for 15 min at 25, 200 *g*. Analysis of GUS activity was done in duplicate for each extraction. The supernatant was

frozen in liquid nitrogen, thawed on ice and 20 µl of supernatant added to 430 µl of pre-heated (37°C) assay buffer (1 mM methylumbelliferyl-beta-D-glucuronic acid (MU), 40 % (v/v) methanol, 60 % (v/v) EB), vortexed briefly and incubated at 37°C. After incubation 100 µl aliquots were added to 1.9 ml stop buffer (0.2 M sodium carbonate) and the product concentration measured with a fluorimeter (Hoefer Scientific Instruments TKO 100) against known concentration standards. The results were compiled with the results of total protein assays to calculate GUS activity (pMol MU min^{-1} mg^{-1} total protein) and corrected for the background level of GUS activity in wild-type tobacco at 10 dpg.

5.2.4 Total protein quantitation

Total protein concentration quantitation of extracts was done in duplicate using the BioRad Protein Dye Reagent, according to the manufacturer's instructions. Optical density (595 nm) was measured using a plate reader (BioRad Plate Reader 450). Measurements were converted to protein concentration by comparison to BSA standards prepared in EB (see section 5.2.3).

5.3 Results

5.3.1 Transgene activity in the progeny of silenced V1:GUSΔC and V2:GUSΔC plants is silenced

An analysis of GUS activity in glasshouse-maintained T_2 progeny from non-silenced and silenced C1:GUS and C3:GUS parent plants showed a level of GUS expression similar to that previously described for each transgenic line (Dry et al. 2000) (Table 5.1). Thus, constitutive expression in the C1:GUS progeny and vascular-limited expression in the C3:GUS progeny was observed. The GUS activity in progeny seedlings derived from a silenced C4:GUS parent also showed the constitutive expression as described previously for this line (Dry et al. 2000) (Table 5.1).

Table 5.1 Transgene activity in progeny TLCV promoter:GUS plants[a]

Transgene	Parent	Progeny expressing GUS[b]	Days post germination[c]
C1:GUS	N[d]	2/2	43
	S[e]	2/2	43
C3:GUS	N	2/2	43
	S	2/2	43
C4:GUS	S	2/2	14
V1:GUSΔC	S	0/2	14
V2:GUSΔC	S	0/5	43

[a]Seeds were collected from TLCV promoter:GUS plants and germinated *in vitro* under antibiotic selection to select for transgenic progeny. After 14 days growth under *in vitro* conditions, seedlings were replanted in soil and maintained in a glasshouse. Histochemical analysis for GUS activity in leaf tissue was done at 14 or 43 days post germination.

[b]Number of progeny plants which tested positive for GUS activity by histochemical assay.
[c]Days post germination at which histochemical assay for GUS activity was done.
[d]Non-infected and non-silenced TLCV promoter:GUS plant.
[e]TLCV-infected and silenced TLCV promoter:GUS plant.

Analysis of V1:GUSΔC progeny seedlings (14 dpg) and V2:GUSΔC progeny plants (43 dpg) derived from silenced parents showed that GUS activity was undetectable in leaf tissue (Table 5.1).

These results show that in progeny derived from silenced TLCV promoter:GUS plants, the expression of GUS activity in C1:GUS, C3:GUS and C4:GUS transgenes was restored. In addition, it was concluded that the silenced state of the V1:GUSΔC and V2:GUSΔC transgenes was inherited.

5.3.2 The inheritance of transgene silencing occurs in the absence of TLCV infection

Geminiviruses have not been shown to be seed-transmissible. To test whether TLCV was seed-borne in these experiments, dot blot hybridisation assays were done to test for the transmission of TLCV infection from parent to progeny TLCV promoter:GUS plants. Dot blot hybridisation analysis of a control tomato plant inoculated with TLCV showed infection. In contrast, TLCV infection could not be detected in the V1:GUSΔC or V2:GUSΔC progeny plants. These results indicated that the silenced state of the V1:GUSΔC and V2:GUSΔC transgenes in progeny derived from silenced parents was not due to TLCV infection, and therefore expression from these transgenes was silenced in the absence of TLCV.

5.3.3 Expression from the V2:GUSΔC transgene is partially restored at 75 days post germination

An extended examination of transgene activity in the progeny of the TLCV promoter:GUS lines (except for the C2:GUS line) was done. Figure 5.1 depicts the experimental approach taken.

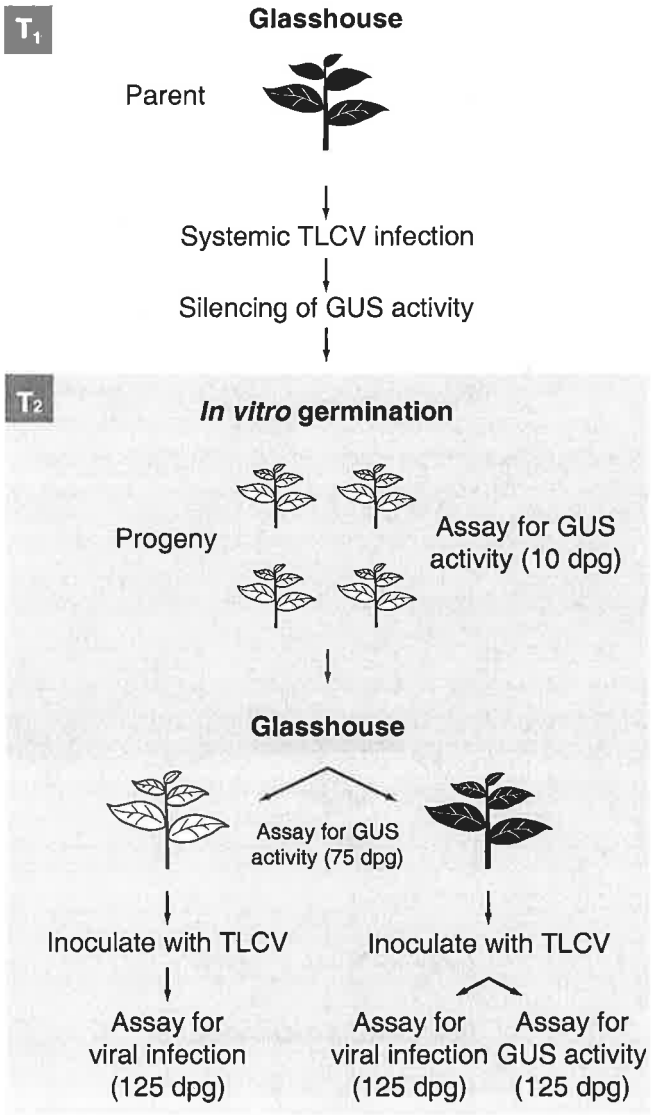


Figure 5.1 Analysis of GUS activity in progeny TLCV promoter:GUS plants. The diagram depicts the approach used to analyse transgene expression in progeny (T_2) plants. Filled plants, positive for GUS activity; unfilled plants, negative for GUS activity. Virus infection was established by agroinoculation. GUS activity was assayed by histochemical analysis of whole seedlings or leaf sections. Seeds were germinated on kanamycin to select for transgenic seedlings. Plants were assayed for virus infection by dot blot hybridisation. dpg, days post germination.

5.3.3.1 Transgene activity in progeny TLCV promoter:GUS plants at 10 dpg

The C1:GUS, C3:GUS and C4:GUS progeny from non-silenced parents generally showed normal patterns of GUS activity (described in section 1.9.2) at 10 dpg (Table 5.2). Although the majority of progeny were positive for GUS activity, a small number of kanamycin-resistant, but GUS-negative plants occurred amongst the progeny TLCV promoter:GUS plants.

The C1:GUS, C3:GUS and C4:GUS progeny from silenced parents were generally positive for GUS activity (Table 5.2), except for 5/5 C4:GUS progeny from the Replicate 1 parent. A further three independent lines of C4:GUS were analysed for GUS activity in the T2 progeny (Lines 2-4, Table 5.2). In each of these lines, the progeny from silenced C4:GUS parents were positive for GUS activity. It was therefore concluded that the silenced phenotype in progeny from the Replicate 1 parent from Line 1 was anomalous, and that GUS activity was restored at 10 dpg in progeny derived from silenced C4:GUS parents.

It was observed by visual analysis that the level of GUS activity in the C1:GUS and C4:GUS progeny from silenced parents appeared to be weaker than in progeny from non-silenced parents. No difference in the level of GUS activity between C3:GUS progeny from non-silenced or silenced parents was observed.

The V1:GUSΔC and V2:GUSΔC progeny from non-silenced parents were positive for GUS activity at 10 dpg. In contrast, GUS activity was not detected in progeny from silenced parents at 10 dpg (Table 5.2). The histochemical GUS assays showed that the silencing occurred throughout the stem and leaves of the seedlings. A further three independent lines of V2:GUSΔC were analysed for GUS activity in T_2 progeny (Lines 2-4, Table 5.2). GUS activity was also not detected in these V2:GUSΔC progeny from silenced parents. Thus, the

Table 5.2 Transgene activity in T_2 TLCV promoter:GUS plants at 10 and 75 days post germination[a]

Transgene	Line	Parent	10 dpg		75 dpg	
			Rep 1[b]	Rep 2[c]	Rep 1	Rep 2
C1:GUS	1	N[d]	5/5	3/5	5/5	4/5
		S[e]	4/5	5/5	2/4	5/5
C3:GUS	1	N	4/5	5/5	5/5	5/5
		S	5/5	5/5	5/5	5/5
C4:GUS	1	N	5/5	5/5	5/5	5/5
		S	0/5	4/4	1/5	6/7
	2	N	4/5	5/5		
		S	5/5	5/5		
	3	N	5/5			
		S	5/5	5/5		
	4	N	5/5			
		S	5/5	5/5		
V1:GUSΔC	1	N	50/50	50/50	5/5	5/5
		S	0/5	0/5	0/5	0/5
V2:GUSΔC	1	N	5/5	5/5	5/5	5/5
		S	0/5	0/5	2/5	
	2	N	5/5	5/5		
		S	0/5	0/5		
	3	N	5/5	5/5		
		S	0/5	0/5		
	4	N	5/5	5/5		
		S		0/5		

[a]T_2 plants were derived and analysed as described in Figure 5.1

[b]Replicate 1
[c]Replicate 2

[d]Non-infected and non-silenced TLCV promoter:GUS plant
[e]TLCV-infected and silenced TLCV promoter:GUS plant

inheritance of the silenced state of the V2:GUSΔC transgene at 10 dpg was consistent in multiple independent lines.

5.3.3.2 Transgene activity in progeny TLCV promoter:GUS plants at 75 dpg

Analysis of GUS activity in the progeny plants was repeated at 75 dpg following the transfer of the plants to a glasshouse. The majority of C1:GUS, C3:GUS and C4:GUS progeny from either non-silenced or silenced parents were positive for GUS activity in leaf tissue (Table 5.2).

GUS activity could not be detected in progeny from silenced V1:GUSΔC parents at 75 dpg. One line of V2:GUSΔC progeny from a silenced parent was analysed at 75 dpg. In contrast to the lack of GUS activity observed at 10 dpg, two out of five V2:GUSΔC plants at 75 dpg were positive for GUS activity. It was observed that the level of GUS activity in the two plants at 75 dpg from a silenced parent was significantly weaker by visual examination than in a control plant from a non-silenced parent at 75 dpg.

Thus, transgene activity in C1:GUS, C3:GUS and C4:GUS progeny from silenced parents was reactivated by 10 dpg, and continued to 75 dpg. However, the level of transgene activity appeared weaker in C1:GUS and C4:GUS progeny from silenced parents compared to progeny from non-silenced parents. The silenced state of the transgenes in silenced V1:GUSΔC and V2:GUSΔC parent plants was inherited by progeny plants at 10 dpg, and continued in V1:GUSΔC progeny at 75 dpg. In contrast, partial restoration of transgene activity occurred at 75 dpg in some V2:GUSΔC progeny from a silenced parent.

5.3.4 TLCV infection induces transgene silencing in the progeny TLCV promoter:GUS plants

The ability of TLCV to infect and to re-establish silencing of transgenes in the progeny TLCV promoter:GUS plants from silenced parents was tested, as depicted in Figure 5.1.

Of the fourteen TLCV promoter:GUS progeny from silenced parents tested at 75 dpg for GUS activity, the three C1:GUS, four C3:GUS and three C4:GUS plants were positive for GUS activity. The three V1:GUSΔC and one V2:GUSΔC plant were negative for GUS activity. These plants were then inoculated with TLCV. Analysis by dot blot hybridisation assay at 50 dpi (125 dpg) showed that each plant was infected with TLCV. Analysis of GUS activity in the C1:GUS, C3:GUS and C4:GUS plants at 50 dpi (125 dpg) showed the silencing of GUS activity. Thus, the silenced state of the V1:GUSΔC or V2:GUSΔC transgenes inherited in plants from silenced parents appeared to have no significant effect on the ability of TLCV to infect. Furthermore, the TLCV promoter:GUS transgenes in plants from a silenced parent were susceptible to silencing following TLCV infection.

5.3.5 The partial restoration of V2:GUSΔC transgene activity occurred in the majority of progeny from a silenced parent

To determine the frequency of partial transgene reactivation in V2:GUSΔC plants from silenced parents (described in section 5.3.3), a second trial was done with 20 plants. Seeds from a silenced T_1 V2:GUSΔC plant were germinated *in vitro* on kanamycin (see section 5.2.2) and tested at 10 dpg for GUS activity (see section 2.2.21). The remaining seedlings were re-planted in soil and maintained in a glasshouse. Leaf tissue from 20 plants was tested at 75 dpg for GUS activity (see section 2.2.21).

GUS activity was not detected at 10 dpg in the V2:GUSΔC progeny. 15 out of 20 V2:GUSΔC plants from a silenced parent showed GUS activity at 75 dpg. A visual comparison with tissue

from a control V2:GUSΔC plant from a non-silenced parent showed that GUS activity in the 15 plants ranged from weak to medium. Expression of GUS in the leaf tissue assayed was either patchy or constitutive. Thus, transgene activity was partially restored in the majority (75 %) of V2:GUSΔC progeny from a silenced parent.

5.3.6 Evidence for a host mechanism in the inherited down-regulation or silencing of GUS activity in progeny TLCV promoter:GUS plants

The heritable but reversible silencing of the V2:GUSΔC transgene was observed in progeny from silenced parents in the absence of TLCV. This suggested that the inherited silenced state of the transgene could be due to epimutation of the transgene mediated by host factors. To assess the effect of chemical modifiers of plant epigenetic regulation on the inherited activity of the V2:GUSΔC transgene, progeny from silenced parents was treated with 5-azacytidine (AzaC), trichostatin A (TSA) or sodium butyrate (SB) and assayed for GUS activity (see section 5.2.3). The experiments described above were also done on C1:GUS progeny.

5.3.6.1 GUS activity in V2:GUSΔC progeny

The effect of the epigenetic modifiers on GUS activity at 10 dpg is shown in Table 5.3. The GUS activity levels in V2:GUSΔC progeny from a non-silenced parent were approximately 80-fold higher than in progeny from a silenced parent. GUS activity in progeny from a silenced parent was near to levels of GUS activity in wild-type tobacco. This result was consistent with the lack of GUS activity observed in V2:GUSΔC progeny assayed histochemically (see section 5.3.3.1). The inclusion of TSA in the germination media of V2:GUSΔC progeny from a silenced parent did not lead to a significant effect on GUS

Table 5.3. The effect of modifiers of epigenetic regulation on transgene activity in T_2 TLCV promoter:GUS plants[a]

Transgene	Parent	Treatment	GUS activity (pmol MU min^{-1} mg^{-1} total protein)
V2:GUSΔC	N[b]		244 ± 30
	S[c]		3 ± 1
	S	AzaC[d]	15 ± 2
	S	TSA[e]	4 ± 1
	S	SB[f]	38 ± 4
C1:GUS	N		1390 ± 65
	S		65 ± 9
	S	AzaC	81 ± 8
	S	TSA	68 ± 5
	S	SB	462 ± 26

[a]T_2 V2:GUSΔC and C1:GUS seedlings derived as described in Figure 5.1 were tested at 10 dpg for GUS activity. Total protein extracts were tested in duplicate by quantitative GUS assay and total protein concentration determined. GUS activities were corrected for background (wild-type tobacco) levels. Activities shown are the average of three independent extractions. Chemical modifiers of epigenetic regulation were added to the germination media as required.

[b]Non-infected and non-silenced TLCV promoter:GUS plant
[c]TLCV-infected and silenced TLCV promoter:GUS plant

[d]5-azacytidine
[e]trichostatin A
[f]sodium butyrate

activity. In contrast, the inclusion of AzaC and SB significantly increased GUS activity to above the baseline activity levels observed in progeny from a silenced parent.

5.3.6.2 GUS activity in C1:GUS progeny

The effect of epigenetic modifiers on GUS activity was also determined for C1:GUS progeny from a silenced parent (Table 5.3). The GUS activity in C1:GUS progeny from a silenced parent was significantly above baseline activity levels, but was only approximately 5 % of the GUS activity observed in C1:GUS progeny from a non-silenced parent. This result was consistent with the visual observation of weaker GUS activity in C1:GUS progeny from silenced progeny compared to progeny from a non-silenced parent (see section 5.3.3.1). The inclusion of TSA in the germination media of C1:GUS progeny from a silenced parent did not have any significant effect on GUS activity. In contrast, the inclusion of AzaC and SB increased GUS activity in C1:GUS progeny from a silenced parent by 19 % and 7-fold, respectively.

The visual observation that GUS activity in the progeny from a silenced TLCV promoter:GUS parent was partially (C1:GUS) or completely (V2:GUSΔC) down-regulated at 10 dpg compared to the GUS activity in progeny from a non-silenced parent was confirmed by a quantitative analysis of GUS activity. The partial reactivation of transgene activity at 10 dpg by AzaC (a non-methylatable cytosine analog) or SB (a histone deacetylase inhibitor) indicated that host mechanisms of epigenetic regulation were involved in the inheritance of the down-regulated (C1:GUS) or silenced (V2:GUSΔC) phenotype in progeny from a silenced parent.

5.3.7 C4:GUS and V1:GUSΔC, but not C3:GUS progeny from a silenced parent show an inherited down-regulation or silencing of GUS activity

An analysis was done to quantify the visual observation that GUS activity (described in section 5.3.3) was lower in C4:GUS and V1:GUSΔC progeny from a silenced parent than in progeny from a non-silenced parent. The results are shown in Table 5.4.

C4:GUS progeny showed a much lower level of GUS activity in progeny from a silenced parent than in progeny from a non-silenced parent. The analysis of V1:GUSΔC progeny showed that GUS activity approached baseline activity levels in the progeny from a silenced parent. These results were consistent with the visual observations that GUS activity in progeny from a silenced parent was partially (C4:GUS) or completely (V1:GUSΔC) down-regulated compared with GUS activity in progeny from a non-silenced parent.

GUS activity in C3:GUS progeny from a silenced parent was higher than in progeny from a non-silenced parent (Table 5.4). This result was in contrast to the results for C1:GUS, C4:GUS, V1:GUSΔC and V2:GUSΔC progeny from a silenced parent but was consistent with the visual observation that the silencing of GUS activity in C3:GUS parents had no effect on progeny GUS activity (described in section 5.2.3).

Table 5.4 Transgene activity in T_2 C3:GUS, C4:GUS and V1:GUSΔC plants[a]

Transgene	Parent	GUS activity (pmol MU min^{-1} mg^{-1} total protein)
C3:GUS	N[b]	98
	S[c]	123
C4:GUS	N	487
	S	84
V1:GUSΔC	N	70
	S	6

[a]T_2 seedlings derived as described in Figure 5.1 were tested at 10 dpg for GUS activity. Total protein extracts were tested in duplicate by quantitative GUS assay and total protein concentration determined. GUS activities were corrected for background (wild-type tobacco) levels. Activities shown are the average of duplicate assays done on one protein extraction.

[b]Non-infected and non-silenced TLCV promoter:GUS plant

[c]TLCV-infected and silenced TLCV promoter:GUS plant

5.3.8 TLCV infection of transgenic tobacco decreased the seed germination rate

In a test of the germination rate of the T_2 seeds taken from the two non-infected and two infected T_1 parent plants of each TLCV promoter:GUS line (as described in section 5.3.3), it was found that seeds from non-silenced plants had an average rate of 92.9 ± 1.2 % (Table 5.5). The average germination rate of seeds from silenced TLCV promoter:GUS plants was 48.6 ± 10.6 %, which was approximately 52 % of the rate observed for seeds from a TLCV-infected plant. The average germination rates between the independent transgenic lines were 90.2 – 96.7 % for seeds from non-silenced plants and 22.7 – 58.3 % for seeds from silenced plants. These results showed that TLCV infection reduced the germination rate of seeds generated by the TLCV promoter:GUS tobacco ($p < 0.025$).

Along with the T_2 TLCV promoter:GUS seeds, T_2 seeds from either a non-infected or TLCV-infected CaMV 35S:GUS tobacco plant (see section 3.3.1) were germinated and the rate recorded at 10 dpg (Table 5.5). The rate of the CaMV 35S:GUS seeds from a TLCV-infected plant was a third of the germination rate observed for seeds from a non-infected plant. This result suggested that the reduction in tobacco seed germination rate following TLCV infection was independent of the transgene sequence carried by the infected tobacco plants.

5.4 Discussion

A previous report of RNA virus-mediated VITGS indicated that silencing was the result of epimutation of the homologous genomic promoter sequence (Jones et al. 2001). The silenced state of the V2:GUSΔC transgene was inherited by progeny plants, which were virus-free. Subsequently, expression from the transgene was restored in the majority of progeny.

Table 5.5 Germination rates at 10 dpg of progeny derived from non-infected and TLCV-infected transgenic tobacco lines[a]

Parent plant		Transgenic line					
		C1:GUS	**C3:GUS**	**C4:GUS**	**V1:GUSΔC**	**V2:GUSΔC**	**35S:GUS**
Non-infected	Number of seedlings	33	29	60	21	19	26
	Number of seeds sown	39	30	62	25	22	26
	Germination rate	84.6 %	96.7 %	96.8 %	84 %	86.4 %	100 %
Non-infected	Number of seedlings	34	28	20	27	29	
	Number of seeds sown	34	29	23	28	20	
	Germination rate	100 %	96.6 %	87 %	96.4 %	100 %	
TLCV-infected	Number of seedlings	8	8	17	25	16	24
	Number of seeds sown	23	32	61	32	32	35
	Germination rate	34.8 %	25 %	27.9 %	78.1 %	50 %	66.6 %
TLCV-infected	Number of seedlings	27	11	4	24	20	
	Number of seeds sown	33	27	23	32	36	
	Germination rate	81.8 %	40.7 %	17.4 %	75 %	55.6 %	

[a]Progeny plants were derived and germinated as described in Figure 5.1

Thus, TLCV-mediated VITGS resulted in the epimutation of the V2:GUSΔC transgene.

In contrast to results obtained with V2:GUSΔC progeny, transgene expression in progeny from silenced C1:GUS and C4:GUS plants was not silenced. Instead, expression from the C1:GUS and C4:GUS transgenes was down-regulated compared to progeny from non-silenced parents (Table 5.3 and Table 5.4). Furthermore, transgene expression was not down-regulated or silenced in C3:GUS progeny from a silenced parent compared to progeny from a non-silenced parent (Table 5.4). Thus, the silenced phenotype of the TLCV promoter:GUS transgenes was either reset (C3:GUS), partially reset (C1:GUS and C4:GUS) or inherited (V1:GUSΔC and V2:GUSΔC) following meiosis. The reason(s) for the differing inheritance patterns between the TLCV promoter:GUS transgenic lines remains unclear. One possibility is that silencing of the larger complementary-sense promoter:GUS transgenes is mediated by a different mechanism(s), which is less efficiently propagated in progeny than the mechanism(s) mediating silencing of the virion-sense promoter:GUS transgenes.

The restoration of transgene activity in mature V2:GUSΔC, but not V1:GUSΔC, progeny from a silenced parent was detected (Table 5.2). Thus, the V1:GUSΔC transgene may have been permanently silenced. Alternatively, the restoration of expression from the V1:GUSΔC transgene may have occurred with further plant growth or in future generations of progeny. Another possibility is that the restoration of expression from the V1:GUSΔC transgene was undetectable by histochemical GUS assay. GUS activity in the mature V2:GUSΔC progeny ranged from weak to medium (see section 5.3.5). However, the level of GUS activity measured in non-silenced T_1 V2:GUSΔC plants is approximately 10-fold more than in V1:GUSΔC plants (Dry et al. 2000). Assuming that GUS activity is approximately proportional to the rate of transcription directed by the V1 and V2 promoters, it is possible that a weak to medium restoration of expression from the V1:GUSΔC transgene was not

detected by the histochemical GUS assay. This hypothesis could be tested by the usage of more sensitive techniques (such as quantitative GUS or RT-PCR assays) to detect expression from the V1:GUSΔC transgene in mature progeny from a silenced parent.

The restoration of V2:GUSΔC expression in mature progeny was to a lower level compared to expression in progeny from a non-silenced parent (see section 5.3.5), suggesting that transgenes silenced by VITGS may require multiple rounds of meiosis before wild-type expression levels are regained. This was also shown by Jones and co-workers (2001) for transgene expression in progeny from a TRV-silenced parent. Transcript levels in T_2 progeny in which expression had been restored were lower compared with a non-silenced plant, as were transcript levels in subsequent T_3 progeny (Fig. 2c, Jones et al. 2001).

Interference with established methylation patterns in silenced V2:GUSΔC progeny plants through AzaC treatment (Table 5.3) suggested that the inherited silencing was at least partially mediated by the methylation of the transgene acquired in the parent plant. This conclusion supported the finding that MET1 (a maintenance CpG methyltransferase) was required for the inheritance of TRV-induced silencing (Jones et al. 2001). AzaC treatment of down-regulated C1:GUS progeny resulted in a smaller, less significant increase in GUS activity compared to V2:GUSΔC progeny, perhaps indicating a more important role for cytosine methylation in the inheritance of the silenced phenotype in progeny.

Hypomethylation of the silenced CaMV 35S promoter following the silencing of MET1 did not result in the complete restoration of transcript levels in progeny from a TRV-silenced parent (Jones et al. 2001). Here, restoration of expression in both C1:GUS and V2:GUSΔC progeny from TLCV-silenced parents was more successful using SB than AzaC (Table 5.3). Taken together, this suggests that a host epigenetic mechanism(s) in addition to cytosine hypermethylation mediates the inheritance of transgene silencing following VITGS. The

effectiveness of SB treatment in the restoration of expression from the C1:GUS and V2:GUSΔC transgenes suggests a role for specific chromatin structure in VITGS.

A divergent response in transgene reactivation was observed using the histone deacetylase inhibitors, TSA and SB (Table 5.3). TSA is reported as a specific HDAC inhibitor which can reactivate silenced mammalian transgenes at nanomolar concentrations (Chen et al. 1997). SB produces many reversible changes in cultured mammalian cells which are proposed to result from, in addition to histone hyperacetylation, changes in chromatin structure and cytoskeleton assembly (Kruh 1982). More recent evidence has shown the ability of SB, but not TSA, to reactivate expression from a mammalian transgene in which silencing was directed by promoter methylation, prompting the proposal of an alternative silencing mechanism operating simultaneously with histone deacetylation in mediating methylation-directed gene silencing (Benjamin and Jost 2001). The absence of transgene reactivation in progeny from TLCV-infected parents treated with TSA suggests that an alternative pathway(s) to histone hypoacetylation may be predominantly responsible for the inherited silencing or down-regulation of transgene activity.

The combination of cytosine hypermethylation following VITGS (Chapter 4) and the SB-mediated restoration of transgene activation in progeny described in this Chapter provides good circumstantial evidence that VITGS of TLCV promoter:GUS transgenes was due to the formation of a local heterochromatic state. Preliminary assays have suggested a higher-order, nuclease-resistant structure was established at the V2:GUSΔC transgene following VITGS (data not shown). Further experiments to determine the higher-order packaging of chromatin at the silenced TLCV promoter:GUS transgenes are warranted.

Histone hypoacetylation was not required for the inheritance of silencing. A histone modification that is more strongly linked to heterochromatin formation is histone H3

methylation (Lachner et al. 2001). Furthermore, a strong biochemical link between histone methylation and CpNpG methylation has been identified in Arabidopsis (Jackson et al. 2002). The identification of CpNpG hypermethylation in the TLCV-derived sequences of the silenced V2:GUSΔC transgene suggests the characterisation of histone modification at the silenced TLCV promoter:GUS transgenes (for example, by chromatin-immunoprecipitation assays) would be informative. In particular, these experiments may shed light on the differing patterns of inheritance between the transgenic lines.

A significant reduction in seed germination was observed following TLCV infection in the TLCV promoter:GUS and CaMV 35S:GUS plants. As discussed in Chapter 3, one explanation for the silencing of the V2:GUSΔC transgene in preanthesis floral tissue may be the replication of TLCV in the floral tissue. This may also explain the reduction in seed germination rate, through a TLCV-mediated disruption in preanthesis floral tissue of cell cycle controls and/or diversion of metabolites to virus propagation, thus reducing the generation or viability of gametophytic tissue. Alternatively, the reduction in seed germination rate may not require replication of TLCV in floral tissue. It is also possible that in non-floral tissues, interference with normal cell cycle controls in infected plant cells could alter nutrient or hormone supply to floral tissues, thus disrupting proper gametophyte development.

The inheritance of the silencing in V2:GUSΔC progeny indicates that the transgene was epigenetically mutated in the embryonic tissue of the parent plant. One possibility is that silencing occurred directly in the embryonic tissue of the infected V2:GUSΔC plant. However, as TLCV is not seed-transmissible and geminiviruses have not been shown to invade embryonic seed tissue (Sudarshana et al. 1998), it is unlikely that this is the case.

Alternatively, TLCV infection and therefore silencing may have occurred in the precursor cells that gave rise to the gametophytic tissues in the infected V2:GUSΔC plant.

Chapter 6

Induction of RNA silencing following TLCV infection

6.1 Introduction

Transcriptional gene silencing induced by RNA viruses is associated with cytosine hypermethylation of the virus-derived genomic sequences (Jones et al. 1999; Jones et al. 2001). This sequence-specific DNA methylation is probably mediated by either DNA-DNA or RNA-DNA interactions in the nucleus (Matzke et al. 2001; Wassenegger et al. 1994). Transcription from a nopaline synthase (NOS) promoter (NOSpro) in Arabidopsis was silenced, following transcription from an unlinked transgene to produce a double-stranded RNA (dsRNA) homologous to the NOSpro sequence (Mette et al. 2000). The transcriptional silencing was associated with hypermethylation of symmetric cytosines in the NOSpro sequence (methylation of asymmetric cytosines was not tested). Silencing and methylation of the NOSpro sequence depended on the synthesis of NOS promoter dsRNA that was degraded to short interfering RNA (siRNA). In this example, DNA methylation was likely to be directed by RNA-DNA interactions. The requirement for siRNA production showed the involvement of the conserved antiviral RNA silencing pathway.

The core of the RNA silencing mechanism can be divided into three components (Voinnet 2001). (1), In most eukaryotic cells, the presence of significant amounts of dsRNA indicates the incursion of a foreign organism. The DICER ribonuclease, which is evolutionarily conserved in drosophila, nematodes, plants and mammals, was able to process dsRNAs to siRNAs (Bernstein et al. 2001; Goldbach et al. 2003). (2), siRNAs associated with proteins to form the RNA-induced silencing complex (RISC), a ribonucleoprotein complex of approximately 500 kiloDaltons (Hammond et al. 2001). (3), RISC was guided by the associated siRNAs to ssRNAs that share sequence homology, which were then degraded to fragments of approximately 25 nt [Hammond, 2000 #274; Voinnet, 2001 #225].

The RNA silencing pathway results in the non-specific degradation of dsRNAs, and the subsequent specific degradation of ssRNAs sharing homology to the dsRNAs. However, transgenes that did not direct transcription of dsRNA were also subject to RNA silencing (Meza et al. 2002). A surveillance process has been proposed that detects aberrant ssRNAs (for example, from transgenes) (Goldbach et al. 2003). Crucial to the RNA silencing of some transgenes is the Arabidopsis gene *sde1*, which is a RNA-directed RNA polymerase (RdRP) (Dalmay et al. 2000). The aberrant RNA may be copied into short dsRNA by the RdRP, which then enters the RNA silencing pathway due to DICER degradation. Thus, some ssRNA species also trigger RNA silencing.

An intriguing aspect of RNA silencing is that it is a non-cell-autonomous process: RNA silencing can be induced locally and then spread to distant sites throughout an organism (Voinnet et al. 1998). Thus, a sequence-specific signal capable of systemic spread is a component of RNA silencing. The signal moves both cell-to-cell and through phloem tissues, resembling the movement of plant viruses (Mlotshwa et al. 2002).

The nature of the systemic signal has not been elucidated, although the sequence-specificity of the signal suggests that it is or has a nucleic acid component. Three RNA species have been proposed as the systemic signal: (1), siRNA; (2), aberrant RNA; (3), larger dsRNA species that are the substrates for DICER (Mlotshwa et al. 2002). Direct evidence for any of the candidates is lacking, although there is good evidence that siRNA is not the signal (Mallory et al. 2001; Tabara et al. 1999).

The strongest evidence that RNA silencing is involved in antiviral defence is that many plant viruses encode proteins that interfere with the RNA silencing pathway. These include the HC-Pro protein of *Potato virus Y* (Anandalakshmi et al. 1998), 2b protein of *Cucumber mosaic virus* (Brigneti et al. 1998), p25 protein of *Potato virus X* (Voinnet et al. 2000) and the AC2 protein of ACMV (Voinnet et al. 1999). Virus suppressor proteins generally have other

replication-related functions and have likely evolved suppressor activity as an additional function. This is reflected by the finding that the suppressor proteins do not share significant homology and interfere with RNA silencing at different points in the pathway (Voinnet 2001).

siRNAs are dsRNA species of 21-27 bp, which can be detected during hybridisation with single-stranded RNA probes of either sense or antisense orientation (Hamilton and Baulcombe 1999). siRNAs are presumed to be synthesised in the cytoplasm and appear to guide dsRNA endonucleases to homologous RNA, thereby targeting it for destruction (Elbashir et al. 2001). Additionally, siRNAs may guide DNA methyltransferases to homologous sequences in the genome (Mette et al. 2000). Two distinct classes of siRNAs have been characterised in plants, the 21-22 nt (short) class and the 25 nt (long) class (Hamilton et al. 2002). Using plant virus-derived suppressor proteins which differentially affected the accumulation of the two siRNA classes, it was demonstrated that the short class of siRNAs were involved in the cytoplasmic degradation of RNA, while the long class of siRNAs were involved in the cytosine methylation of nuclear DNA sequences. Thus, siRNAs produced in the cytoplasm may enter the nucleus to mediate the sequence-specific methylation of homologous sequences.

An example of the detection of plant virus-specific siRNAs has been described. siRNAs complementary to the genomic(+) RNA strand of PVX were identified during infection of *N. benthamiana* (Hamilton and Baulcombe 1999). The aim of the work described in this chapter was to determine whether TLCV-specific siRNAs were produced during infection in solanaceous host plants and to determine if TLCV-specific siRNAs were candidates to direct the DNA methylation associated with TLCV-mediated VITGS. Total RNA from infected plants was extracted and probed with TLCV RNA probes. siRNAs homologous to the genic

sequences of TLCV were detected in four solanaceous host species. siRNAs homologous to the intergenic sequences of TLCV were detected in tobacco. Furthermore, siRNAs were detected in tobacco that were homologous to the V2:GUSΔC transgene sequence which became hypermethylated in silenced V2:GUSΔC plants.

6.2 Materials and methods

6.2.1 Analysis of small interfering RNA

Three plants from each of four solanaceous species (*N. tabacum, N. benthamiana,* tomato and Datura) were inoculated with TLCV (see section 2.2.20). Small expanding leaves from each plant were harvested at 7 and 21 dpi along with leaves from non-infected plants. Leaves were also sampled from tobacco at 14 and 28 dpi.

Total RNA was extracted from the leaf samples (see section 2.2.13.1; leaf samples from 7 dpi were pooled together before RNA extraction). Fractionation, transfer and detection of siRNA by hybridisation was done by the method of Wang and co-workers (2001) with the following modifications. Denaturing PAGE electrophoresis (see section 2.2.2.2) was done with 10 μg of total RNA was from each plant. The transfer of RNA to nylon membrane was done in 0.5 X TBE (see Table 2.1) using a Mini-PROTEAN 2 electrophoresis system with a Mini Trans-Blot cell (BioRad, USA). Hybridisation was done at 42°C in a formamide buffer (50 % (v/v) deionised formamide, 125 mM Na_2PO_4 (pH 7.2), 250 mM NaCl, 7 % (w/v) SDS). Membranes were placed on wet Whatman paper (2 X SSC, Table 2.1), wrapped in plastic wrap and exposed to Biomax film (Kodak, USA) at -70°C in a film cassette containing a Biomax intensifying screen (Kodak, USA).

Membranes were stripped of hybridised ^{32}P-labelled RNA by the addition of boiling 0.5 X SSC, 0.5 % SDS buffer. The membranes were incubated at room temperature with occasional agitation for 0.5 h, rinsed for 30 s with 2 X SSC and allowed to dry at room temperature. The

membranes were exposed to film overnight as described above to check for the absence of signal.

As required, the RNA was digested with a mixture of RNases before electrophoresis. 10 μg of RNA was digested by the addition of 2 μg of RNase A and 1 μg of RNase T_1 for 30 min at room temperature. The product was then fractionated by polyacrylamide gel electrophoresis. Digestions were done at low salt concentration (10 mM Tris.Cl, 1 mM EDTA) thus ensuring the sensitivity of dsRNA to Rnase A digestion.

6.3 Results

6.3.1 TLCV-specific siRNAs accumulate during TLCV replication in tobacco

Evidence for the induction of the plant RNA silencing antiviral pathway during TLCV infection was assessed by the detection of TLCV-homologous siRNA in infected plants.

Hybridising nucleic acids homologous to the 3'-end of the TLCV V2 ORF sequence were detected in TLCV-infected tobacco plants (Fig. 6.1b). Hybridisation signals were detected in the samples taken at 21 dpi but were not detected in samples taken from non-infected plants or at 7 dpi. RNase digestion of the RNA from tobacco at 21 dpi and the subsequent loss of hybridisation signal confirmed that the observed signals were due to the hybridisation of the ^{32}P-labelled probe to RNA in the infected plant (Lane "R", Fig. 6.1b).

The hybridising RNAs migrated to a position between the 21 and 27 nt DNA oligonucleotide markers and were detected using a probe homologous to the virion-sense strand of the 3'-end of the V2 ORF (Probe 1, Fig. 6.1a). Hybridising RNAs were also detected in the tobacco plants at 21 dpi using a probe homologous to the complementary-sense strand of the 3'-end of the V2 ORF sequence (Upper panel, Fig. 6.2). Thus, the TLCV-specific hybridising RNAs in tobacco plants at 21 dpi were double-stranded.

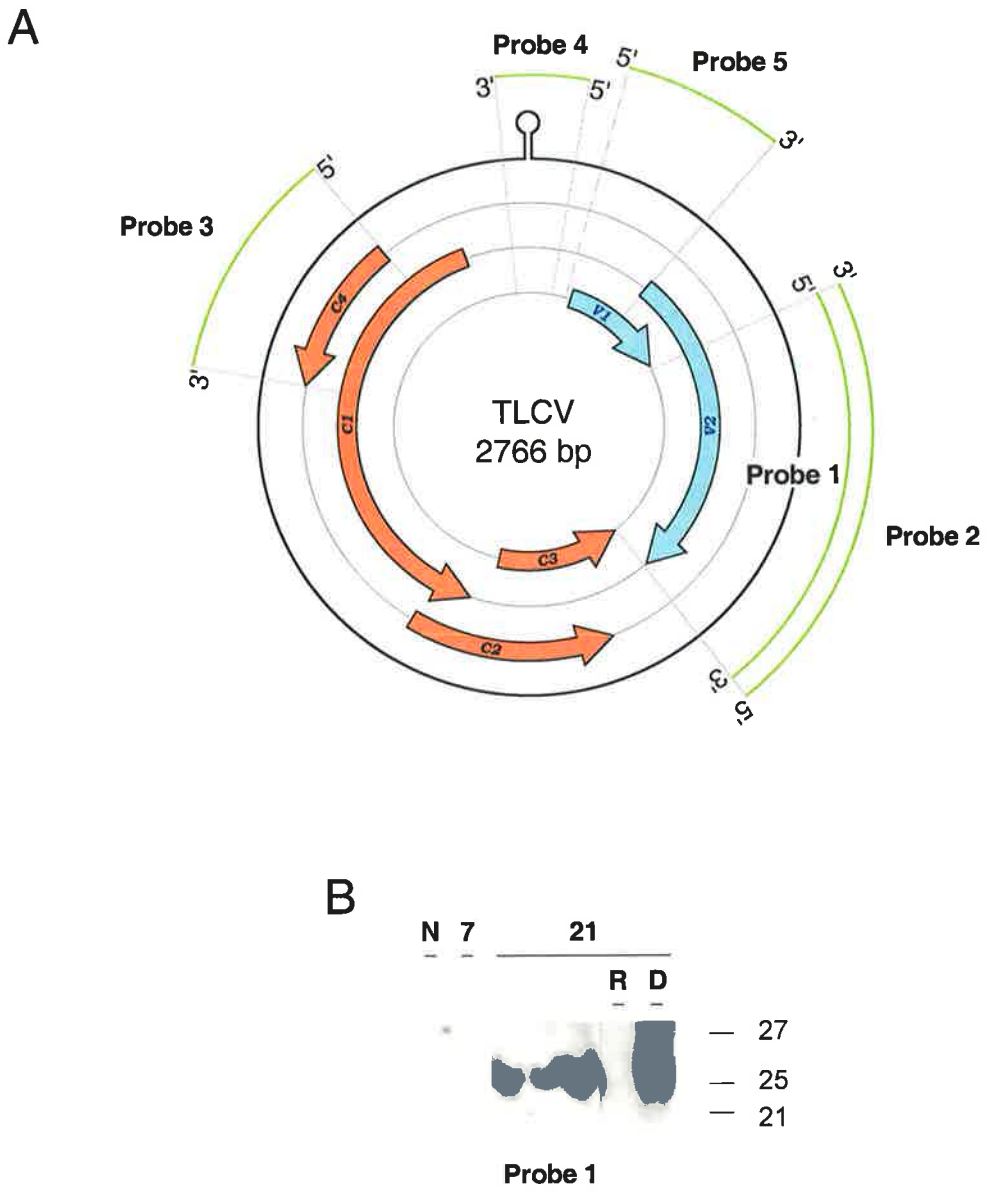


Figure 6.1 Accumulation of TLCV-specific siRNAs during infection of tobacco with TLCV. siRNAs homologous to the 3'-end of the V2 ORF were detected in tobacco plants at 21 days post inoculation (dpi). (A), Diagram of TLCV replicative form (RF) DNA showing the location of the six virus ORFs. The position of the single-stranded RNA probes used for the detection of siRNA in this study are shown by the green bars. The RNA probes were homologous to either the virion- (5'-3') or complementary-sense (3'-5') strands of the TLCV RF DNA. The TLCV co-ordinates of the probes are as follows. Probes 1 and 2: 492-1075; Probe 3: 2156-2464; Probe 4: 2718-91; Probe 5: 138-325. (B), Hybridisation of a probe homologous to the virion-sense strand of the TLCV RF DNA (Probe 1) to RNA from tobacco infected with TLCV. DNA oligonucleotides of 21, 25 and 27 nt were used as size markers. N: RNA from non-infected plant; 7: RNA from plant at 7 dpi; 21: RNA from plant at 21 dpi; R: RNase treatment; D: DNase treatment. Plants were inoculated with TLCV via *Agrobacterium* and samples collected at 7 and 21 dpi.

Hybridising nucleic acids homologous to the 3'-end of the TLCV V2 ORF sequence were detected in TLCV-infected tobacco. The dsRNA nature of the nucleic acids confirmed that they were TLCV-specific siRNAs. Thus, TLCV infection of tobacco resulted in the accumulation of TLCV-specific siRNAs.

6.3.2 TLCV-specific siRNAs accumulate in other TLCV hosts

The accumulation of TLCV-specific siRNAs in tobacco indicated the induction of the tobacco RNA silencing pathway during TLCV infection. The induction of the RNA silencing pathway in other TLCV host species was assessed by the analysis of TLCV-specific siRNA during infection.

TLCV-specific siRNAs homologous to the 3'-end of the V2 ORF sequence were detected in TLCV infected tobacco, *N. benthamiana*, tomato and *D. stramonium* (Upper panel, Figure 6.2). siRNAs were detected in the samples from each species at 21 dpi but were not detected in samples taken from non-infected plants or at 7 dpi (not done for *D. stramonium*).

The intensity of the siRNA signal was strong in tobacco, *N. benthamiana* and *D. stramonium* samples, in contrast to the weak intensity of the signal in the tomato samples. Analysis of the ethidium bromide-stained RNA species that migrated into the polyacrylamide gel indicated that the amount of total RNA loaded for all samples was approximately equal (Lower panel, Figure 6.2).

Thus, siRNAs homologous to the TLCV V2 ORF were detected in all four solanaceous species infected with TLCV. The siRNAs accumulated at the highest level in tobacco and at the lowest level in tomato.

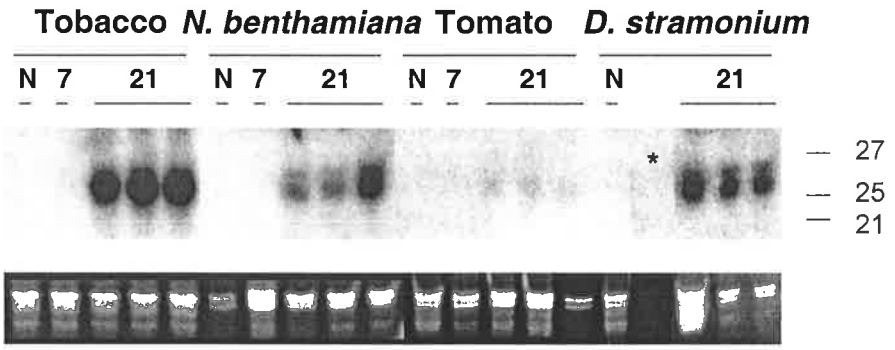


Figure 6.2 Accumulation of TLCV-specific siRNAs during infection of various Solanaceous species with TLCV. siRNAs homologous to the 3'-end of the V2 ORF were detected in tobacco, *Nicotiana benthamiana*, tomato and *Datura stramonium* at 21 days post inoculation (dpi) using Probe 2 (Figure 6.1A) (upper panel). DNA oligonucleotides of 21, 25 and 27 nt were used as size markers. N: RNA from non-infected plant; 7: RNA from plant at 7 dpi; 21: RNA from plant at 21 dpi. *: not done. Lower panel shows the eithidium bromide-stained gel indicating the relative loading of the samples used in the upper panel. Plants were inoculated with TLCV via *Agrobacterium* and samples collected at 7 and 21 dpi.

6.3.3 siRNA homology extends to the TLCV complementary-sense ORFs and intergenic region

Virus-specific siRNAs are presumed to be the degradation products of virus RNAs that have been processed by the RNA silencing pathway (Voinnet 2001). TLCV-specific siRNAs homologous to a virion-sense ORF were detected in infected tobacco (see section 6.3.1). However, TLCV also encodes actively-transcribed ORFs in the complementary-sense orientation (Mullineaux et al. 1993). The accumulation of siRNAs homologous to the TLCV complementary-sense ORFs and IR was assessed in tobacco.

siRNAs homologous to the C1/C4 ORFs were detected in TLCV-infected tobacco at 21 dpi (Fig. 6.3a). The siRNAs were detected in samples taken at 21 dpi but were not detected in samples taken from non-infected plants or at 7 dpi.

siRNAs homologous to the TLCV IR were detected in TLCV-infected tobacco at 21 dpi (Figs. 6.3b). The siRNAs were detected in samples taken at 21 dpi but were not detected in samples taken from non-infected plants or at 7 dpi. This region of homology extended between (but did not include) the mapped C1 and V1 ORF transcriptional start points located in the TLCV IR, respectively (Mullineaux et al. 1993). The susceptibility of the IR-specific siRNAs to RNase digestion confirmed that the hybridisation signals observed in Figure 6.3b were due to the probe hybridising to RNA in the infected tobacco (data not shown).

Thus, siRNAs homologous to TLCV virion- and complementary-sense sequences which are transcribed and translated during infection were detected in tobacco, as were siRNAs homologous to untranslated sequences of TLCV.

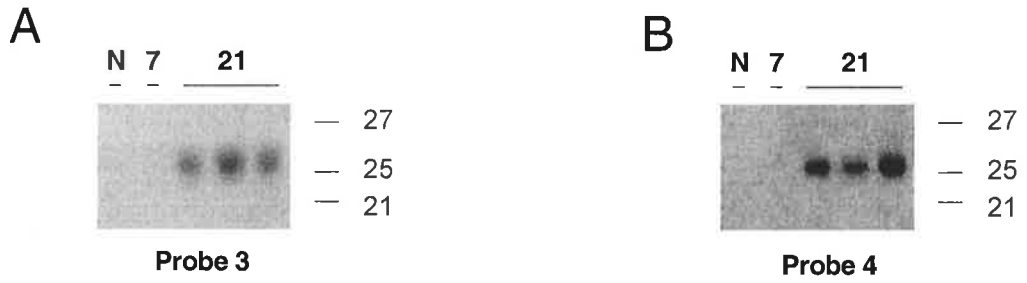


Figure 6.3 Accumulation of siRNAs homologous to the TLCV complementary-sense ORFs and intergenic region (IR) during infection of tobacco with TLCV. siRNAs homologous to the C4 ORF or a fragment of the IR encompassing the TLCV origin of replication were detected in tobacco plants at 21 days post inoculation (dpi). (A), Hybridisation of a probe homologous to the TLCV C4 ORF (Probe 3, Fig. 6.1A) to RNA from tobacco infected with TLCV. (B), Hybridisation of a probe homologous to the TLCV IR (Probe 4, Fig. 6.1A) to RNA from tobacco plants infected with TLCV. DNA oligonucleotides of 21, 25 and 27 nt were used as size markers. N: RNA from non-infected plant; 7: RNA from plant at 7 dpi; 21: RNA from plant at 21 dpi. Plants were inoculated with TLCV via *Agrobacterium* and samples collected at 7 and 21 dpi.

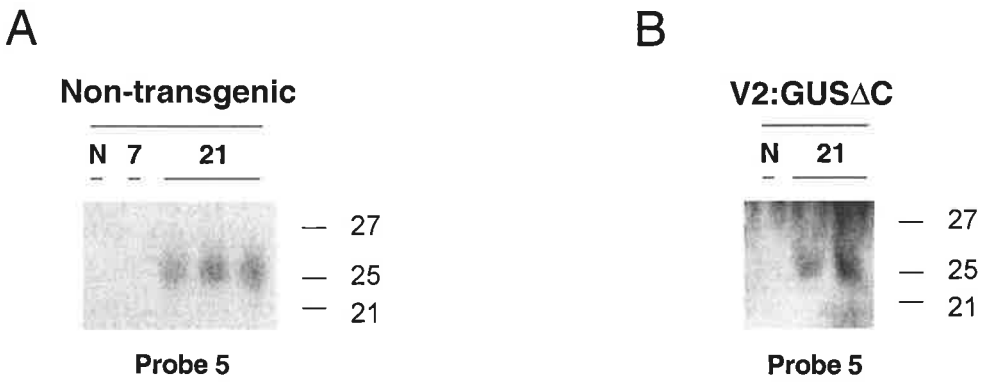


Figure 6.4 Accumulation of siRNAs homologous to the TLCV sequences upstream of the V2 ORF during infection of non-transgenic and V2:GUSΔC tobacco during TLCV infection. siRNAs were detected in tobacco plants at 21 days post inoculation (dpi) using a probe homologous to the 5'-ends of the TLCV V1 and V2 ORFs. (A), Hybridisation of Probe 5 (Fig. 6.1A) to RNA from non-transgenic tobacco infected with TLCV. The tobacco membrane shown in Figure 6.2 was stripped and hybridised with Probe 5. (B), Hybridisation of Probe 5 to RNA from V2:GUSΔC tobacco plants infected with TLCV. DNA oligonucleotides of 21, 25 and 27 nt were used as size markers. N: RNA from non-infected plant; 7: RNA from plant at 7 dpi; 21: RNA from plant at 21 dpi. Plants were inoculated with TLCV via *Agrobacterium* and samples collected at 21 dpi.

6.3.4 siRNAs specific to the 5'-untranslated region of the TLCV V2 ORF transcript accumulate during infection

TLCV-induced silencing of the V2:GUSΔC transgene is associated with cytosine hypermethylation of the TLCV-derived transgene sequences upstream of the GUS ORF (see section 4.3.2.1), which includes the long untranslated region of the TLCV V2 transcript (Mullineaux et al. 1993). The accumulation of siRNA homologous to this sequence was assessed in non-transgenic and V2:GUSΔC tobacco.

siRNAs homologous to the 5'-ends of the TLCV V1 and V2 ORFs were detected in TLCV-infected non-transgenic and V2:GUSΔC tobacco at 21 dpg (Fig. 6.4a and Fig. 6.4b). This region of homology extended from three bp upstream of the V1 ORF transcriptional start point to 17 bp downstream of the start of the V2 ORF (see Fig. 1.2a for description of transcription of the V1 and V2 ORFs). The siRNAs were not detected in non-infected non-transgenic or V2:GUSΔC plants, or in infected non-transgenic tobacco at 7 dpi.

The TLCV sequence (co-ordinates 138-325) used as the RNA probe was also found directly upstream of the GUS ORF in the V2:GUSΔC transgene. This sequence in the V2:GUSΔC transgene was hypermethylated following VITGS. Thus, siRNAs homologous to the TLCV-derived regions of the V2:GUSΔC transgene which were hypermethylated in silenced plants accumulated following TLCV infection of both transgenic and non-transgenic plants.

6.4 Discussion

The hallmark of the antiviral mechanism known as RNA silencing is the production of virus-specific siRNAs during infection (Mlotshwa et al. 2002). The analysis of siRNA during TLCV infection in tomato, *N. benthamiana*, *D. stramonium* and tobacco revealed the production of TLCV-specific nucleic acids that migrated between DNA oligonucleotides of

21 and 27 nt (Fig. 6.2). The double-stranded nature of the nucleic acids was confirmed by using either TLCV virion-sense (Probe 1, Fig. 6.1a and Fig. 6.1b) or complementary-sense (Probe 2, Fig. 6.1a and Fig. 6.2) strand-specific probes for the analysis of infected tobacco. The nucleic acids detected in infected plants were confirmed as RNA by RNase and DNase treatment of tobacco samples before analysis (Fig. 6.1b). Thus, the detection of TLCV-specific siRNAs showed that the induction of RNA silencing occurred in multiple host species during TLCV infection.

The reason for the low accumulation of siRNAs in tomato compared to siRNA accumulation in tobacco (Fig. 6.2) is not readily apparent. One possibility is that RNA silencing was induced more efficiently following infection in tobacco than in tomato. Alternatively, the minimal accumulation of siRNAs in tomato may have been due to TLCV-mediated interference in the tomato RNA silencing pathway at a point between induction and siRNA production. Comparison of the TLCV titre in tobacco and tomato plants at equivalent developmental stages may be useful in addressing this hypothesis.

Results presented in Chapters 3 and 4 have led to the postulation that TLCV-induced transcriptional silencing may be mediated by a mobile silencing signal carrying a nucleic-acid specificity determinant. The characteristics of the proposed signal include systemic spread into mesophyll tissue and the ability to induce sequence-specific DNA methylation of the TLCV promoter:GUS transgenes in non-infected cells. The siRNAs detected in infected non-transgenic and V2:GUSΔC tobacco (Figs. 6.4a and 6.4b) are ideal candidates to mediate the sequence-specific methylation of the V2:GUSΔC transgene described in Chapter 4. It is tempting to speculate that the mobile silencing signal is equivalent to the sequence-specific systemic signal of RNA silencing. In this scenario, the RNA silencing systemic signal generated is exported from TLCV-infected cells and induces the production of TLCV-specific

siRNAs in non-infected cells, which then direct methylation of the TLCV promoter:GUS transgenes (Hamilton et al. 2002). Sequence alignment of TLCV (between co-ordinates 2354 and 149) and TLCV D1, ACMV or TYLCV (see Appendix 1) reveal that consecutive sequences of nucleotide homology of 25 nt or more occur only between TLCV and TLCV D1, supporting the hypothesis of siRNA-directed TLCV promoter:GUS transgene methylation.

The virus proteins p25 and 2b suppress RNA silencing by preventing the movement of the systemic signal out of virus-infected cells (Mlotshwa et al. 2002). However, the HC-Pro virus suppressor protein suppresses the synthesis of siRNAs, but does not prevent the movement of the systemic signal (Mallory et al. 2001). If TLCV-induced silencing is indeed mediated by RNA silencing, the observed suppressor activity of the TLCV C2 gene product (Selth et al. 2003) must not prevent systemic spread of the signal. This hypothesis is supported by the movement of a silencing signal induced by TGMV infection in *N. benthamiana* into non-infected cells (Peele et al. 2001). Yet, the TLCV C2 gene product clearly did not prevent the accumulation of siRNAs during TLCV infection. Taken together, these observations suggested that the process by which geminivirus suppressor proteins interfere with RNA silencing was unique amongst the characterised virus suppressor proteins.

The majority of plant RNA viruses replicate via a dsRNA intermediate (Hull 2002), thus producing a potent target for the DICER component of RNA silencing. The trigger for RNA silencing during DNA virus infection is uncertain (Voinnet 2001). Bi-directional transcription of geminivirus dsDNA could lead to the synthesis of sense and antisense transcripts with overlapping sequences (Mullineaux 1992), thereby producing a dsRNA trigger.

Alternatively, TLCV may trigger RNA silencing in host plants by virus RNAs being detected as aberrant, thereby generating TLCV dsRNAs by the action of host RdRP. Intriguingly, a virus RdRP can direct transcription of RNA from a ssDNA template (Siegel et al. 1999). Host RdRP transcription from TLCV ssDNAs may result in the synthesis of partial or full-length complementary-sense TLCV transcripts. Further RdRP activity could produce large dsRNA targets for DICER. The suggested role of RdRP in TLCV RNA silencing could be tested by the mutation of the tobacco homolog of *sde1/sgs2* (Dalmay et al. 2000).

siRNAs specific to both virion-sense and complementary-sense ORFs of TLCV were identified in infected tobacco (see section 6.3.1). A reasonable assumption is that transcripts of the TLCV virion-sense and complementary-sense ORFs provide the trigger for the production of siRNAs specific to these virus sequences. siRNAs specific to a sequence (TLCV co-ordinates 2718-91) between the mapped TSS of the TLCV IR (Fig. 1.2, the TSSs at co-ordinates 2715 and 141) were also detected. Thus, the fragment of the TLCV IR that has not been previously shown to be transcribed was able to trigger RNA silencing and the subsequent production of siRNAs.

A number of hypotheses to explain this result can be put forward. As suggested above, TLCV–induced RNA silencing could involve host RdRP-directed transcription of virus ssDNAs. Transcription of the IR may provide the trigger leading to the production of IR-specific siRNAs.

A second possibility involves the identification of a consensus TATA box promoter element located on the complementary-sense strand of the TLCV IR and beginning at co-ordinate 118 (Fig. 1.2 and (Dry et al. 1993)). No evidence is available that indicates transcription is initiated from this TSS, however the complementary-sense TATA box sequence is identical to the virion-sense TATA box sequence that likely directs transcription of V1 and V2

(Mullineaux et al. 1993). According to the scanning model of translation, initiation from the TSS at co-ordinate 118 would transcribe an ORF between co-ordinates 88-2750 to produce a polypeptide of 35 amino acids. This transcript would include the TLCV stem-loop sequence motif and therefore be capable of forming a hairpin structure, thus perhaps presenting a susceptible target for the DICER component of the RNA silencing pathway (Mette et al. 1999). A third possibility involves the occurrence of read-through transcription from the TLCV genome. The TLCV transcripts 3'-ends have been mapped to polyadenylation sequences located between the C3 and V2 ORFs (Mullineaux et al. 1993). The possibility remains of read-through transcription of either the virion- or complementary-sense strands to generate transcripts containing IR sequences. Such aberrant transcripts may then enter the RNA silencing pathway, resulting in the production of siRNAs homologous to both the genic and non-genic sequences of TLCV.

Chapter 7

General Discussion

7.1 Summary of results and conclusions

The work described in this thesis was undertaken with the broad aims of furthering current knowledge of geminivirus biology and the understanding of processes in the development of TLCV infection. The results presented in this thesis characterise the homology-dependent gene silencing response of transgenic tobacco lines following TLCV infection. Here, a summary of results and conclusions are presented. This is followed by a discussion of the implications of the results for TLCV biology and the development of TLCV resistance strategies.

Dry and co-workers (2000) have described a series of transgenic tobacco lines in which expression of a reporter GUS gene is driven by promoter sequences derived from TLCV. In contrast to expectations, TLCV infection of these plants did not result in the transactivation of transgene expression. Instead, the silencing of GUS activity in each transgenic line was observed.

The characterisation of the parameters for TLCV promoter:GUS transgene silencing following TLCV infection are described in Chapter 3. Silencing of the TLCV promoter:GUS transgenes was shown to be sequence- and virus-specific. TLCV infection resulted in the silencing of transgenes carrying promoter sequences derived from TLCV, but not CaMV. One transgenic line, V2:GUSΔC, was used as a representative of the TLCV promoter:GUS lines. Silencing of the V2:GUSΔC transgene occurred following infection by TLCV (and a TLCV strain) but not the heterologous geminiviruses ACMV or TYLCSV. Silencing of the V2:GUSΔC transgene did not require the TLCV C3, C4 or V1 gene products. Wholemount *in situ* hybridisation experiments to localise TLCV DNA in infected V2:GUSΔC leaf tissue, along with unpublished *in situ* hybridisation results from this laboratory (S. Rasheed, manuscript in preparation), indicated that TLCV was restricted to replication in host vascular

tissue. However, silencing of the TLCV promoter:GUS transgenes occurred in both vascular and mesophyll tissue.

Characterisation of the biochemical changes that occurred following the silencing of the TLCV promoter:GUS transgenes are described in Chapters 3 and 4. Nuclear run-on analysis showed that silencing of the V2:GUSΔC transgene occurred at the level of transcription. This finding was in agreement with the lack of detectable GUS transcripts in silenced C1:GUS and V2:GUSΔC plants using RT-PCR. Bisulfite modification and sequencing was used to assess the cytosine methylation of the V2:GUSΔC transgene in silenced tissue. Following TLCV infection, the TLCV-derived sequences of the transgene became hypermethylated at both symmetric and asymmetric cytosine positions. In contrast, the GUS ORF sequences of the V2:GUSΔC transgene became slightly hypomethylated. The sequence-specific methylation and transcriptional silencing of the TLCV promoter:GUS transgenes following TLCV infection represented the first description of VITGS associated with a geminivirus infection.

To further characterise the mechanism(s) mediating silencing, transgene expression was examined in the progeny of silenced and non-silenced TLCV promoter:GUS plants (Chapter 5). The silenced phenotype observed in TLCV-infected V2:GUSΔC plants was inherited in virus-free progeny. Subsequently, the partial restoration of transgene activity was observed in the majority of silenced V2:GUSΔC progeny with further plant growth. Thus, TLCV infection resulted in the epimutation of the V2:GUSΔC transgene. The silenced phenotype was also inherited by progeny of silenced V1:GUSΔC plants. In contrast, the silenced phenotype of infected complementary-sense TLCV promoter:GUS plants was either partially (C1:GUS and C4:GUS) or completely (C3:GUS) reset in progeny. Treatment using the epigenetic modifier chemical, AzaC, indicated that inheritance of the silenced or down-regulated phenotype in progeny was at least partially mediated by patterns of cytosine methylation established in the parent plants. Additionally, interference with inherited chromatin structures in progeny from

silenced parents indicated that non-hypoacetylation mediated heterochromatin formation may be important for the inheritance of VITGS.

siRNAs, a characteristic component of the HdGS phenomenon known as RNA silencing, are reported to direct sequence-specific methylation of nuclear sequences in plants (Hamilton et al. 2002). The possibility that siRNA production was associated with TLCV-mediated VITGS was assessed (Chapter 6). siRNAs specific to a virion-sense ORF sequence of TLCV were detected in non-transgenic tobacco, tomato, *N. benthamiana* and datura plants. This showed that the host antiviral mechanism of RNA silencing was induced following infection by TLCV. siRNAs homologous to the TLCV-derived V2:GUSΔC transgene sequences which became hypermethylated following VITGS were detected in non-transgenic and V2:GUSΔC tobacco following TLCV infection. Thus, TLCV-specific siRNAs were a candidate for the mechanism directing sequence-specific methylation of the V2:GUSΔC transgene. Unexpectedly, siRNAs specific to a fragment of the TLCV IR were detected in tobacco. This result raises question as to the nature of the RNA silencing trigger during DNA virus infections in plants.

7.2 TLCV-mediated VITGS and RNA silencing

VITGS is associated with *de novo* methylation of the silenced 'target' nuclear sequences, which is required for inheritance of the silenced phenotype in progeny (Jones et al. 1999; Jones et al. 2001). In this study, evidence has been obtained that heterochromatin formation at the target nuclear sequences may also be involved in VITGS (Chapter 5). Biochemical pathways have been identified by which DNA methylation can direct the local formation of heterochromatin (see section 5.4). Furthermore, a candidate (siRNA) to mediate the sequence-specific methylation associated with VITGS was identified (Chapter 6). If VITGS is indeed associated with RNA silencing, the question therefore arises as to whether siRNA-directed

DNA methylation is sufficient to induce the local assembly of heterochromatin. siRNAs were reported to be crucial for directing histone H3 methylation (a hallmark of heterochromatin) in a sequence-specific manner in the fission yeast *Schizosaccharomyces pombe* (Volpe et al. 2002). Thus, while this pathway has not been characterised in plants, evidence exists that siRNAs may be to direct sequence-specific heterochromatin formation. It is also possible that siRNA-directed heterochromatin formation may precede siRNA-directed DNA methylation, or that DNA methylation directly results from siRNA-directed heterochomatin formation.

7.3 Asymmetric silencing: nuclear, but not virus, sequences are silenced during TLCV-mediated VITGS

The results presented in Chapter 6 show that the antiviral mechanism of RNA silencing was induced following TLCV infection. Unfortunately, although this response was conserved amongst multiple host species, it is apparent that RNA silencing was not able to eliminate virus infection. However, preliminary evidence indicated that the RNA silencing pathway in tobacco may be able to limit the level of TLCV infection (data not shown). It is likely that TLCV combats the RNA silencing mechanism through the suppressor activity of the TLCV C2 gene product (Selth et al. 2003; Voinnet et al. 1999). A strategy to overcome C2-mediated interference in RNA silencing may be useful in boosting the efficacy of this natural antiviral response to geminivirus infection.

Geminiviruses, once having gained entrance to the nucleus of host cells, convert their single-stranded DNA genomes to a double-stranded replicative form, which is then assembled into a mini-chromosome, most likely using host histones (Pilartz and Jeske 1992). Thus at some stages of the geminivirus replicative life-cycle, the viral DNA closely resembles the host genome. If VITGS was mediated by DNA methylation and/or heterochromatin formation, it is puzzling that TLCV was not subject to silencing. *In vitro* methylation of geminivirus DNA

inhibited virus propagation (Brough et al. 1992). Furthermore, plants are able to methylate episomal replicating virus DNA (Tang and Leisner 1998). If VITGS is mediated by DNA methylation, this suggests that geminiviruses are either able to avoid methylation of their genomes by host machinery, or that propagation of unmethylated geminivirus genomes is positively selected for. Alternatively, VITGS could be mediated by heterochromatin-mediated transcriptional repression. This may result in a nuclear pool of non-silenced and silenced virus copies, allowing infection may continue through the positive selection of non-silenced copies. Alternatively, the geminivirus mini-chromosome may not be available to host chromatin modelling factors, or the mini-chromosome structure may not be amenable to heterochromatinization, thus precluding heterochromatin-mediated silencing.

7.4 TLCV-induced transcriptional gene silencing as a tool

VITGS associated with TLCV infection resulted in the silencing of homologous transgene promoter sequences (Chapter 3). The silenced state of the promoter sequence could be inherited in progeny (Chapter 5). It remains to be seen whether the insertion of endogenous host promoter sequences in TLCV would result in the heritable silencing of the host promoter copy following infection. The development of TLCV as a promoter silencing vector would likely be of some use in plant molecular biology, given the ease with which geminivirus genomes can be modified and inoculated into host plants (Peele et al. 2001).

TLCV-induced transcriptional gene silencing places a restriction on the use of TLCV promoters to drive plant transgene expression in areas infected with the virus, as the engineered plant phenotype would likely be lost following infection. Thus, this consideration must be taken into account during the design of host resistance strategies employing TLCV promoter sequences. One possible resistance strategy could be based on the engineering of a hypersensitive response in host plants, whereby local cell death is triggered by the TLCV-

mediated silencing of homologous promoter sequences. However, if VITGS does indeed have a systemic silencing component as proposed in Chapter 3, this strategy may be limited by the triggering of cell death in non-infected local and systemic tissue.

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Appendix 1

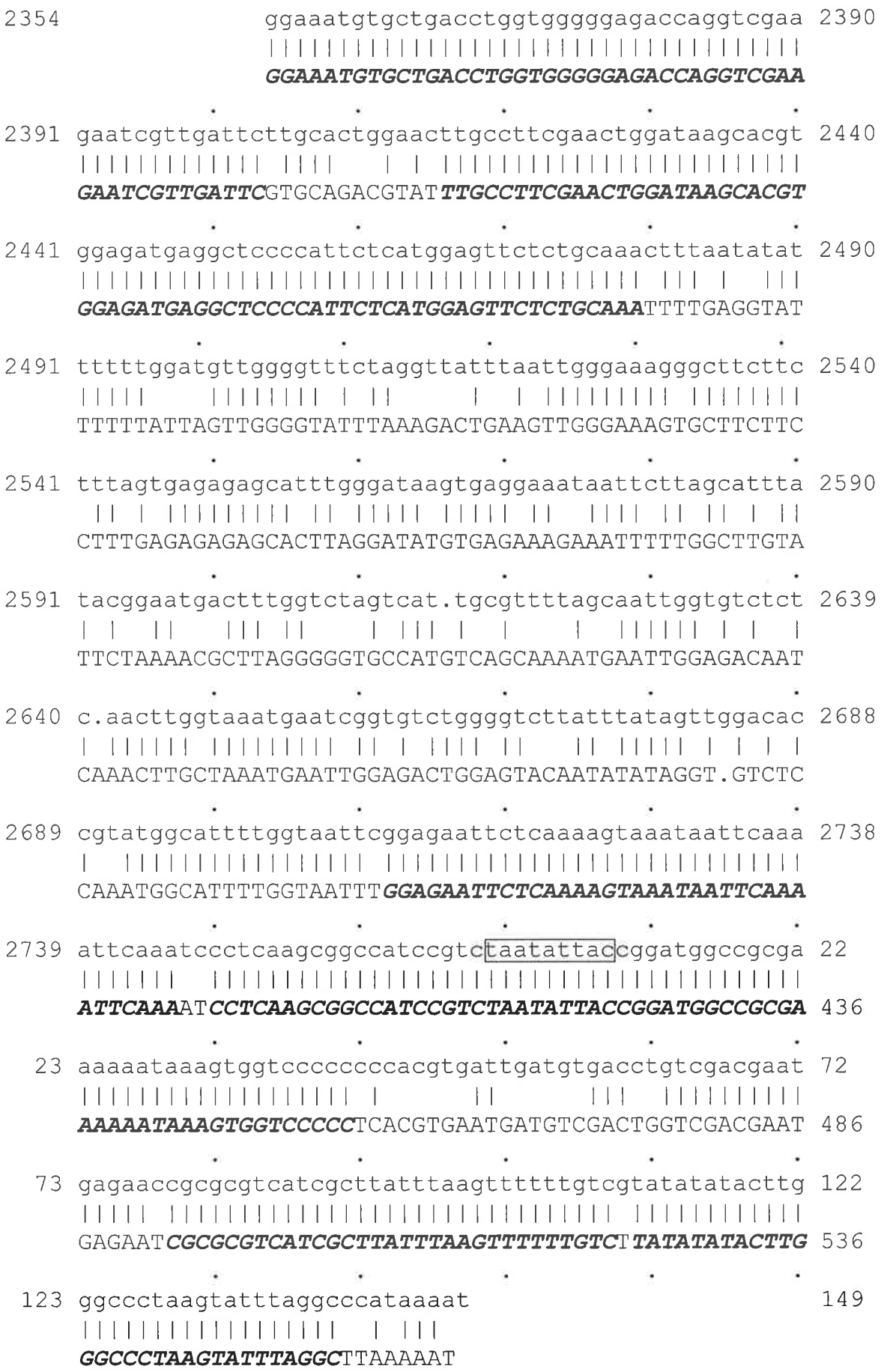

Figure A1.1. Alignment of TLCV and TLCV D1 nucleotide sequences. TLCV sequences between co-ordinates 2354 and 149 (Dry et al. 1993) were aligned against TLCV D1 sequences using the software program GAP (www.angis.com). The geminivirus consensus nonanucleotide sequence is shown within the open box. Consecutive TLCV D1 sequences of 25 nt or more showing homology to TLCV are highlighted in bold italic.

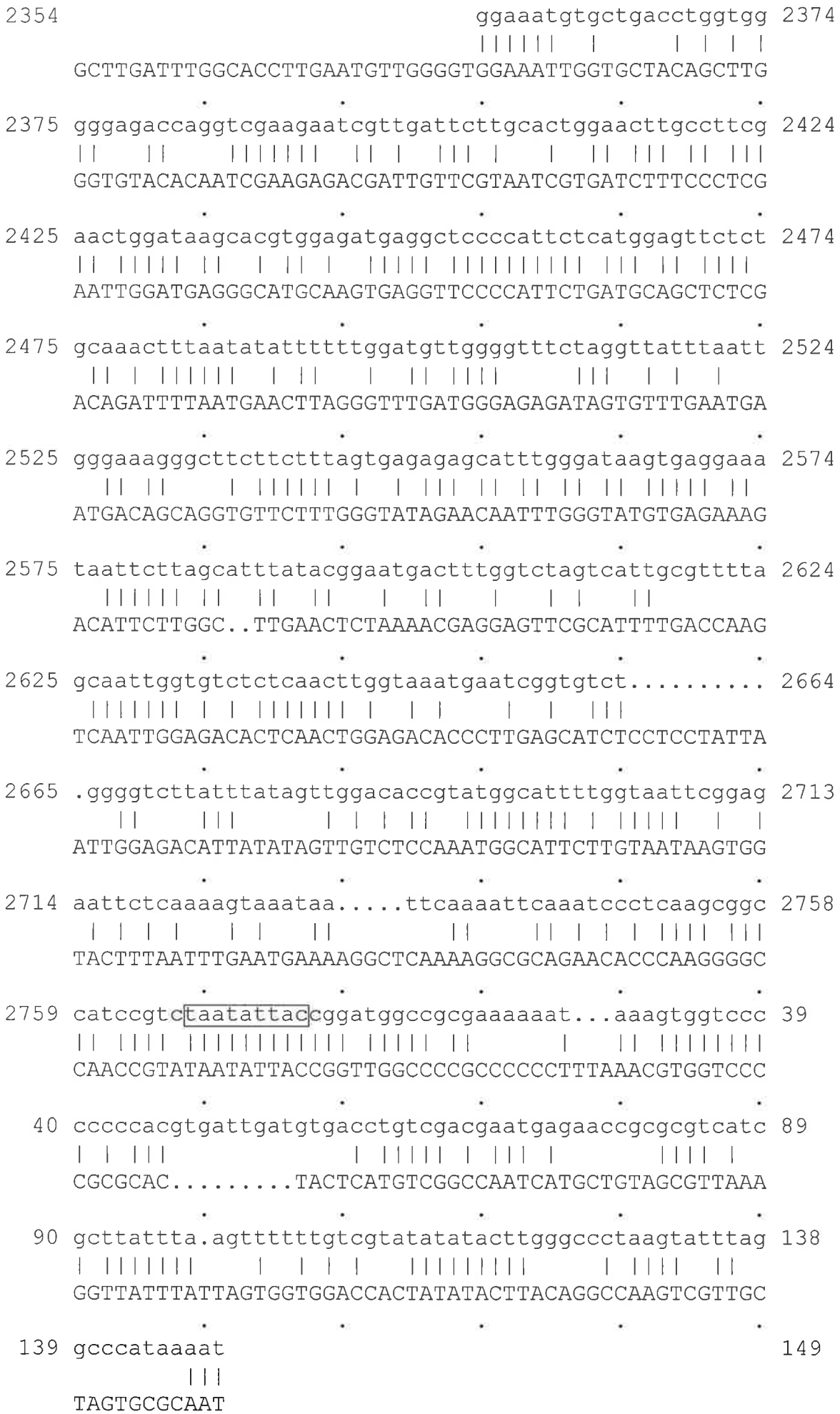

Figure A1.2. Alignment of TLCV and ACMV nucleotide sequences. TLCV sequences between co-ordinates 2354 and 149 (Dry et al. 1993) were aligned against ACMV sequences using the software program GAP. The geminivirus consensus nonanucleotide sequence is shown within the open box.

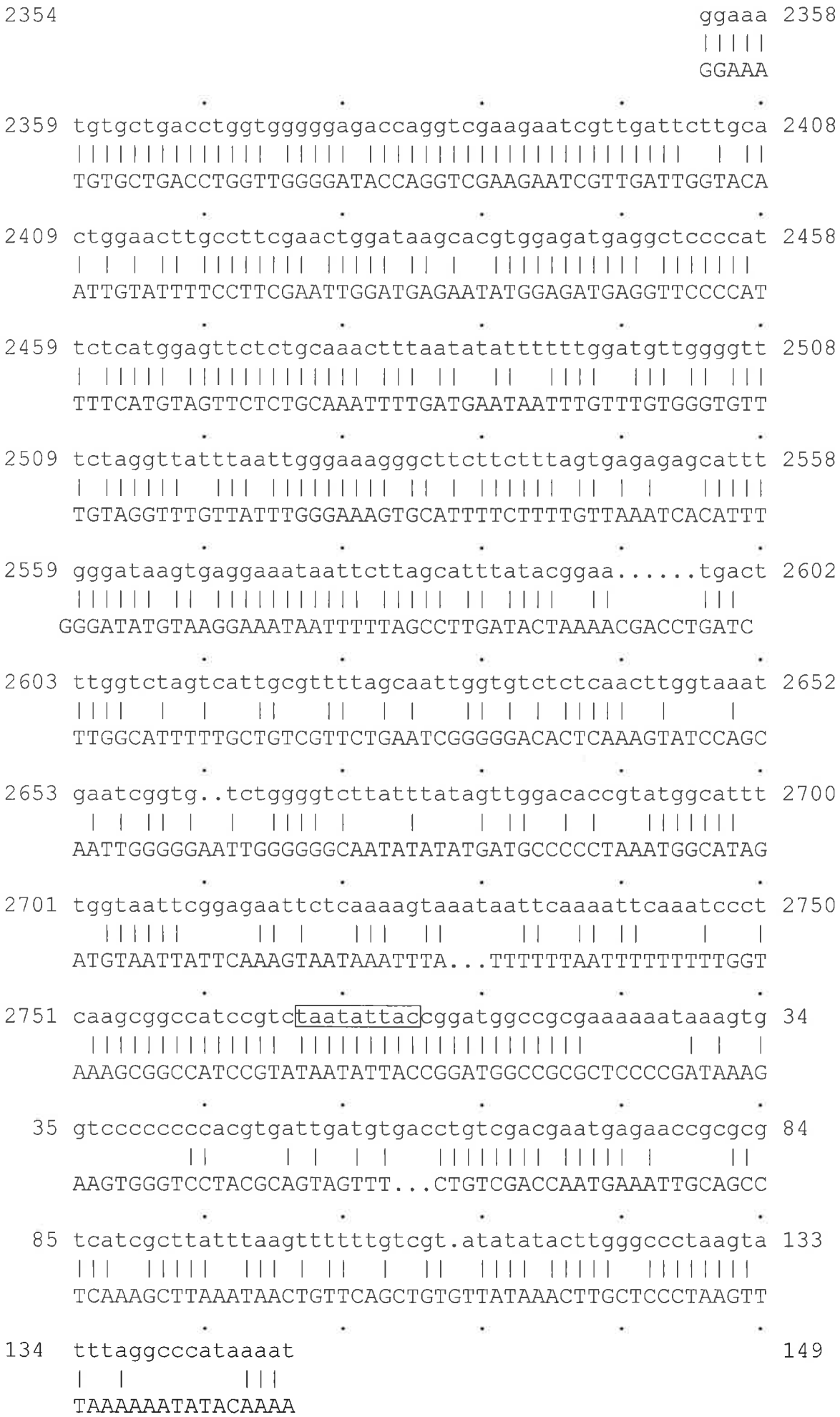

Figure A1.3. Alignment of TLCV and TYLCSV nucleotide sequences. TLCV sequences between co-ordinates 2354 and 149 (Dry et al. 1993) were aligned against TLCV D1 sequences using the software program GAP. The geminivirus consensus nonanucleotide sequence is shown within the open box.

Appendix 2

Questions from the Examiner's Reports not requiring modification to this thesis. Examiner's questions/comments are stated, followed by reasons why thesis modification is not warranted.

Examiner 1

Comment 2. "I realise that ToLCV is first acknowledged in the thesis and then TLCV is used. Please consider using a global find and replace to switch to ToLCV. It's hard to get used to, but in a few years papers using TLCV will appear outdated."

Response: The publication arising from the body of work presented in this thesis has utilised TLCV as the abbreviation for *Tomato leaf curl virus*. For the sake of continuity between this thesis, the publication arising and the publication by Dry et al. (MPMI, 2000, 13:529-537) on which this body of work is based, the abbreviation TLCV will be retained.

Question 4. "Table 5.2. There is evidence that temperature can have a major impact on silencing and virus infection (Szittya et al. 2003 EMBO 22:633-640). If possible, please comment as to whether you think differences between rep 1 and rep 2 infected plants at 75 dpg could be due to different temperatures. Were the experiments done at the same time of year?"

Response: Szittya et al. (2003) report that siRNA accumulation following virus-induced induction of RNA silencing in plants is abrogated at low temperatures (i.e. 15°C). However, with increasing temperatures the induction of RNA silencing is reactivated. The replicate experiments producing the results shown in Table 5.2 were done simultaneously and therefore

under the same conditions. Additionally, as all plant growth experiments were done in a temperature-controlled glasshouse, temperature variation between the replicate plants in unlikely to explain the variation in transgene activity between plants observed. Furthermore, while possible, the initial maintenance of the silenced phenotype in progeny is unlikely to be due to RNA silencing as the silencing trigger (TLCV replication) is absent in these plants.

Comment 5. "It was misleading to the reviewer to refer to infected plants as silenced and non-infected plants as non-silenced. Perhaps using U/N (uninfected, non-silencing) and I/S (infected, silencing) could be used instead."

Response: While it is perhaps initially confusing to refer to TLCV-infected and transgene-silenced plants as silenced, Chapter 3 firmly establishes in the reader's mind this link, especially as no instances where TLCV infection did not result in transgene silencing were reported.

Using abbreviations like those suggested by Reviewer 1 would require modification during discussion of results regarding T_2 progeny (Chapter 5 and later) which inherit silencing in the absence of infection, making comparison between transgene expression in parent and progeny plants cumbersome and confusing. Furthermore, the nomenclature employed in thesis and in another report of similar experiments using the *Tobacco rattle virus* (Jones et al., 2001, Current Biology 11:747-757) was also used in the publication directly arising from this body of work.

Comment 7. "Table 3.1. It would be useful to show an alignment of the ToLCV and ToLCV strain D1 sequences. There is abundant evidence that at least 21 (26) or more consecutive nucleotides must show homology to trigger silencing. The conclusion from this table, that 84

% homology is enough to produce silencing in 100 % of the plants is not really well supported. It did seem that 2-3 plants were used to make this conclusion. If that is not the case, please make that clear."

Response: Sequence alignments of TLCV D1, ACMV and TYLCSV are presented in Appendix 1. A comment about the alignments has been inserted into the General Discussion.

Question 8. Table 5.5. What is the germination efficiency of ToLCV-infected, non-transgenic tobacco."

Response: The germination efficiency of TLCV-infected wild-type tobacco was not tested and therefore cannot be properly commented upon. It can be noted that discussions between the author and Mr. L. Krake (CSIRO Plant Industry, Urrbrae, South Australia) indicated that a reduction in seed germination following TLCV infection of various Solanaceous hosts was common. However, this needs to be experimentally verified.

Examiner 2

Question 4. "p. 54, line 4. Is 50 dpi a relevant time period for plant infection? What happens at earlier times? Any promoter activation? (need to look at V1:GUS or V2:GUS, not Δ C versions)."

Response: 50 dpi corresponds to the late stages of plant infection. However, the results at 50 dpi presented are more concerned with the level of transgene activity in the presence of replicating virus. Here, 50 dpi simply represents the time-point at which transgene activity is fully abolished in the plants tested. As TLCV titres were not tested before 50 dpi, it cannot be

discounted that the level of virus replication was altered in the presence of the transgenes undergoing silencing. However, the comparison of TLCV DNA accumulation in non-silenced CaMV 35S:GUS plants and silenced plants at 50 dpi (Fig. 3.1) suggests that this is not the case.

The activation of integrated TLCV promoters during the early stages of TLCV infection is an interesting question. The use of agroinoculation or whitefly transmission as the sole methods to cause TLCV infection make quantitative testing of transgene activity in whole plants during the early stages of infection difficult. A better system would be to test promoter activity in protoplasts derived from the promoter:GUS plants following transfection with infectious TLCV constructs.

Comment 9. "Should discuss biological relevance- particularly with respect to time- frames of virus replication vs silencing: also lack of effect on virus titres and symptom development."

Response: the points noted are dealt with in the General Discussion (Chapter 7).

Question 11. "Couldn't it also be true that it's a mixed population of ssRNA of each sense? Relative resistance of dsRNA to RNase?"

Response: The results presented in Chapter 6 do not rule out the detection of short ssRNA of each sense. It is an outstanding question in the field of homology-dependent gene silencing whether siRNAs are actually double-stranded *in vivo*. However, the mechanism by which siRNAs are proposed to direct sequence-specific RNA degradation and DNA methylation is

based on the base-pairing ability of single-stranded molecules of siRNA. The important point is that siRNAs of both sense are detected, which is demonstrated here.

Question 12. "Fig 6.1 Denaturing or non-denaturing gels?"

Response: The use of denaturing gel electrophoresis is stated in section 6.2.1.

Seemanpillai, M., Dry, I., Randles, J. & Rezaian, A. (2003).Transcriptional silencing of geminiviral promoter-driven transgenes following homologous virus infection. *Molecular Plant-Microbe Interactions, 16*(5), 429-438.