Tissue targeting signals of

Tomato leaf curl virus

by

Muhammad Saif-ur-Raisheed
M. Sc. (Hons), The University of Agriculture, Faisalabad, Pakistan

A thesis submitted for the degree of
Doctor of Philosophy

in

The Faculty of Sciences
The University of Adelaide,
School of Agriculture, Food and Wine,

in collaboration with

CSIRO Plant Industry,
Horticulture Unit

Urrbrae, Adelaide
July, 2006

to my family

Table of contents

Abstract

Tomato leaf curl virus (TLCV; family *Geminiviridae*, genus *Begomovirus*) causes a severe viral disease of cultivated tomato in northern Australia. Geminiviruses are economically-important plant pathogens and possess either one or two circular, single-stranded DNA (ssDNA) genomes each of 2.5-3 kb. Geminiviruses have been reported to replicate in, and localise to, the nuclei of host plant cells. The tissue and intracellular distribution of the monopartite TLCV was investigated by *in situ* hybridization. Contrary to the previous understanding of geminiviral localization, single-stranded (ss) DNA of TLCV accumulated in the cytoplasm. TLCV ssDNA was also found in the nucleus, as were lower levels of replicative form double-stranded (ds) DNA. Under the same conditions, *Tomato golden mosaic virus* (TGMV) ssDNA and dsDNA were found in nuclei. ssDNA of TLCV, TGMV and *Tomato yellow leaf curl Sardinia virus* (TYLCSV) was detected in some xylem vessels under specific hybridization conditions. Phloem specificity of TLCV was partially released by co-infection with TGMV.

To understand the mechanism of TLCV movement, infectious constructs containing a disrupted V1, V2 or C4 ORF were analysed by their localization at the cellular level. The V1 and C4 mutants were able to spread systemically, but less efficiently than wild-type virus. Transgenic plants expressing C4 complemented the C4 mutant virus, allowing it to spread systemically at the same rate as the wild-type virus. The V2 mutant was unable to infect plants systemically, but when bombarded onto a detached leaf was found to replicate in target cells without infecting the neighbouring cells. To characterize the functions of V1, V2 and C4 in TLCV movement, each ORF was

fused to the green fluorescent protein (GFP) and their subcellular localisation was analysed. V1:GFP exhibited a nuclear and cell peripheral localisation, and was probably associated with the endoplasmic reticulum. V2:GFP was targeted to nuclei and accumulated in nucleoli. C4:GFP was observed at the cell periphery in a punctate pattern consistent with plasmodesmatal (Pd) localisation. Plasmolysis of the transiently expressing V1:GFP and C4:GFP cells suggested an association of these viral proteins with Pd. *N. benthamiana* epidermal cells transiently expressing GFP:V1 and GFP:C4 when treated with Brefeldin A (BFA) showed disrupted distribution of GFP suggesting that V1 and C4 proteins were targeted to the cellular periphery via the Golgi apparatus. Using an inducible system it was found that localization of C4:GFP to cellular membrane was rapid.

To understand the role of a host factor in virus movement, subcellular localization of SlUPTG1:GFP fusion protein was studied. SlUPTG1 is a protein closely related to a family of plant reversibly glycosylated peptides and recently in our laboratory it has been shown that it interacts with V1. SlUPTG subcellular localization resembled the localization of V1:GFP where it was mainly associated with cell wall components. Furthermore, the expression of *SlUPTG1* in a transient TLCV replication assay increased the accumulation of viral DNA, suggesting that this host factor plays a role in viral infection. Together, these results have shown that three viral proteins (CP, V1 and C4) are involved in cell-to-cell and long distance movement, and that a host factor has a possible role in viral movement.

RNA silencing is a sequence-specific mechanism regulating gene expression and has been used successfully for antiviral defense against RNA viruses. Similar strategies to develop resistance against DNA containing TLCV and some other geminiviruses

have been unsuccessful. To determine the fate of a transgene carrying homology to the virus, transgenic plants carrying the TLCV *C4* gene, which induces a distinct phenotype, were used. Upon TLCV infection, the phenotype was abolished and the *C4* transcript disappeared. Concurrently, TLCV specific siRNAs were produced. *In situ* hybridization showed abundant levels of TLCV DNA in phloem cells of TLCV-infected *C4* transgenic plants. However, the *C4* transcripts were no longer detectable in non-vascular cells. Analysis of the transgene by methylation sequencing revealed a high level of *de novo* methylation of asymmetric cytosines in both the *C4* ORF and its 35S promoter. A high level of methylation was also found at both symmetric and asymmetric cytosines of the complementary-sense strand of TLCV double-stranded DNA. Given the previous finding that methylated geminiviral DNA is not competent for replication, a model whereby TLCV evades host defence through a population of *de novo* synthesized unmethylated DNA is proposed.

Satellite ßDNA molecules are becoming an emerging threat to global agriculture by their association with an increasing number of begomoviruses. Tissue specificity of a satellite DNA ß molecule associated with *Cotton leaf curl virus* (CLCuV) was identified. Both DNA ß and CLCuV were found to be phloem specific. Furthermore, it was found that DNA ß transreplicates with TLCV in phloem tissues. However, in contrast DNA ß did not transreplicate with TGMV, which is a mesophyll invasive virus.

This work has provided a new insight into the biology of TLCV showing a distinct pattern of localization and mechanism of movement compared with a bipartite begomovirus. Furthermore, the spread of silencing to non-infected tissues upon TLCV infection and escape of TLCV from silencing explains why transgenic resistance is

inconsistent for geminiviruses. Given that SlUPTG1 has a role in TLCV movement; down-regulation of *SlUPTG1* may provide a durable resistance by blocking the virus movement.

Declaration

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

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

Muhammad Saif-ur-Raisheed

July 2006

Acknowledgements

I would like to thank my supervisor, Ali Rezaian, for his support to my application of John Allwright Fellowship and providing me a chance to work in his group at CSIRO. In addition to his excellent guidance, I have respected his friendship and caring attitude. I also thank to John Randles, my principal supervisor, for his assistance and encouragement in my project and his valuable expertise of plant virology. I am indebted to Anna Koltunow, my co-supervisor, for her assistance in carrying out the *in situ* experiments and many other molecular biology techniques in her group. Anna I am greatly thankful to you for your help during my studies and also for designing the future projects after my studies. I am grateful to the Australian Centre for International Agricultural Research (ACIAR) for awarding me a John Allwright Fellowship to complete my studies in the University of Adelaide and CSIRO Plant Industry for providing me the facilities to carry out my project.

I am grateful to Yusuf Zafar for his constant support at NIBGE and for giving a freshly graduated student a chance to work in his group, and then encouraging me to work on a tissue culture project which at one stage seemed to likely fail. Due to Yusuf Zafar's support, I was able to start my PhD studies at the Adelaide University.

I would like to extend my thanks to all of the members of the virology group at CSIRO Plant Industry. In particular, I greatly appreciate the methylation work of Xue-Yu Bian which significantly contributed to the understanding of silencing mechanism discussed in chapter 6. I am also grateful to Luke A Selth for his consistent support in the lab and reading drafts of different manuscripts, and with whom I collaborated to perform the

GFP studies. Luke and I shared a couple of manuscripts together. Many thanks to Susan Johnson who helped me to develop *in situ* hybridization technique, along with other microscopic techniques. Susan without your help it was difficult to establish *in situ* technique and to get exciting results. Thanks to Jamus and Karina for their assistance in the lab. Karina managed to keep the laboratory consumables available whenever needed and organised almost everything around the lab. More broadly, I would like to thank all the staff at CSIRO Plant Industry in Adelaide for being very generous with their time and resources. Specifically I would like to mention Mandy Walker who was always happy to spare time for scientific discussions. Also, thanks to Mrs Maria Piscioneri for her administrative support, Mrs Julie Powell who did her best to ensure I had everything necessary for my work.

I am grateful to Shunhong Dai and Roger N Beachy from Donald Danforth Laboratories in the US for providing the tobacco BY-2 cell culture and putting lots of efforts to make transgenic BY-2 cell lines, Nam-Hai Chua from Rockfeller University, USA for providing the pTA7002 plasmid, John Stanley for providing the infectious clones of TGMV, Bruno Gronenborn for providing the infectious clones of TYLCSV, B. Loveys for statistical analyses, Ghafar Sarvestani for assistance with the confocal scanning laser microscope, Angela Renfry and Meredith Wallwork for helping with tissue identification. .

I thank my family, particularly my Mum Jee and friendly Abbu Jee, and my three lovely sisters for their support and love. Abbu Jee and Chachoo Hamid, I really appreciated your financial support that has allowed me to become who I am today. I thank to my late grandmas who always prayed for my success. Thanks to friends around Adelaide

especially Muhammad Saeed, Sagar Saeed and Kashif Majeed and friends back in Pakistan, for their sincere company, happy distractions and life beyond the science.

Finally I want to thank my wonderful beloved wife Zarqa for her boundless love, understanding and support. Most importantly to my lovely Son, Muhammad Afnan who really suffered by our PhDs (me and my wife's PhD) and we were not able to give him the time for which he really deserves. I am sorry Afnan for not attending some of your assemblies at school, I love you.

Abbreviations

AbMV	*Abutilon mosaic virus*
ACMV	*African cassava mosaic virus*
ADK	adenosine kinase
AMP	adenosine monophosphate
AP	alkaline phosphatase
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
AYVV	*Ageratum yellow vein virus*
BCIP	5-bromo-4-choloro-3-indolylphosphate
BCTV	*Beet curly top virus*
BDMV	*Bean dwarf mosaic virus*
BFA	Brefeldin A
BGMV	*Bean golden mosaic virus*
bp	base pairs
c4	TLCV mutant containing mutation in *C4* ORF
CaMV	*Cauliflower mosaic virus*
cDNA	complementary DNA
CLCuMV	*Cotton leaf curl Multan virus*
CM	common motif
CP	coat protein
CS	complementary-sense
CSR	complementary-strand replication
DAPI	4', 6-diamidino-2-phenylindole dihydrochloride

DEPC	diethyl pyrocarbonate
DEX	Dexamethasone
DIC	differential interference contrast
DIG	digoxygenin
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
dpi	days post-inoculation
DRM	domains rearranged methylase
dsDNA	double-stranded DNA
dsRNA	double-stranded RNA
EDTA	ethylenediamine-*tetra*-acetic acid
ER	endoplasmic reticulum
EuMV	*Euphorbia mosaic virus*
EtOH	ethanol
g	gram(s)
g	relative centrifugal force
GFP	green fluorescent protein
GUS	ß-glucuronidase
h	hour(s)
HRP	horseradish peroxidase
IPTG	*Iso*-propyl-β-D-thiogalactopyranoside
IR	intergenic region
ISH	in situ hybridization
kb	kilobase pairs
L	litre(s)
LB	Luria broth

M	molar
mA	miliampere
min	minute(s)
MOPS	3-N-Morpholinopropanesulfonic acid
MP	movement protein
mRNA	messenger RNA
MSV	*Maize streak virus*
NBT	4-nitroblue tetrazolium chloride
NLS	nuclear localisation signal
NSP	nuclear shuttle protein
NTP	nucleoside triphosphate
nt	nucleotide
ORF	open reading frame
ori	origin of replication
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
Pd	Plasmodesmata
PLRV	*Potato leaf roll virus*
PM	Plasma membrane
pRBR	plant retinoblastoma-related protein
PTGS	post-transcriptional gene silencing
PVDF	polyvinylidene fluoride
RB	retinoblastoma protein
RCNMV	*Red clover necrotic mosaic virus*
RCR	rolling circle replication
RdDM	RNA-directed DNA methylation

rDNA	ribosomal DNA
RDR	recombination-dependent replication
REn	replication-enhancer protein (encoded by *C3* or *REn*)
Rep	replication-associated protein
RF	replicative form
RGP	reversibly glycosylated peptide
RISC	RNA-induced silencing complex
RNA	ribonucleic acid
rpm	revolutions per minute
rRNA	ribosomal RNA
RYMV	*Rice yellow mottle virus*
s	second(s)
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
siRNA	small interfering RNA
SqLCV	*Squash leaf curl virus*
SSC	standard sodium citrate
ssDNA	single-stranded DNA
ssRNA	single-stranded RNA
TAR	transcriptional activation region
TEMED	N, N, N'- N'- Tetramethylethylenediamine
TBE	tris-borate-EDTA
TGMV	*Tomato golden mosaic virus*
TGS	transcriptional gene silencing
TLCV	*Tomato leaf curl virus* (Australian isolate)
TMV	*Tobacco mosaic virus*

ToLCNDV	*Tomato leaf curl New Delhi virus*
Tris	tris(hydroxymethyl)aminomethane
TYLCV	*Tomato yellow leaf curl virus*
TYLCCNV	*Tomato yellow leaf curl China virus*
TYLCSV	*Tomato yellow leaf curl Sardinia virus*
UAS	upstream activating sequence
V	volt(s)
v1	TLCV mutant containing mutation in *V1* ORF
v2	TLCV mutant containing mutation in *V2* ORF
VIGS	virus-induced gene silencing
VS	Virion-sense
WDV	*Wheat dwarf virus*
WT	wild-type
YFP	yellow fluorescent protein

Publications

Sections of this thesis have been published in the following articles (see Appendix 3):

Journal articles:

a) Published

1 Selth, L. A., Dogra, S. C., **Rasheed, M. S.**, Healy, H., Randles, J. W., and Rezaian, M. A. (2005). A NAC Domain Protein Interacts with Tomato leaf curl virus Replication Accessory Protein and Enhances Viral Replication. *Plant Cell* **17**(1), 311-325.

2 **Rasheed, M. S**, Selth, L. A., Koltunow, A. M. G., Randles, J. W., and Rezaian, M. A. (2006). Single-stranded DNA of the vascular-associated *Tomato leaf curl virus* accumulates in the cytoplasm. *Virology* **348** (1), 120-132

3 Xue Yu B., **Rasheed, M. S.**, Seemanpillai, J. M., Rezaian, M. A. (2006). The role of DNA methylation in *Tomato leaf curl virus* escape of host silencing. *Mol. Plant Microbe. Interact.* **19**(6), 614-624

4 Selth, L. A., Dogra, S. C., **Rasheed, M. S.**, Randles, J. W., and Rezaian, M. A. (2006). Identification and characterization of a host reversibly glycosylated peptide that interacts with the *Tomato leaf curl virus* V1 protein. *Plant Mol. Biol.* **61** (1-2), 299-312

b) In preparation

5 **Rasheed, M. S.**, Eini Gandomani, O., Koltunow, A. M. G., Randles, J. W., and Rezaian, M. A. (2006). Satellite DNA β molecule is phloem specific and transreplicate with the phloem specific helper virus. To be submitted to *Virus Research*. Internal review.

6 **Rasheed M. S.**, Johnson, S., Okada, T., Hu Yingko, Rezaian, M. A., and Koltunow, A. M. G. (2006). Double labelling *in situ* hybridization and laser capture microdissection using plastic embedded plant tissues. To be submitted to *BioTechniques*. Internal review.

7 **Rasheed, M. S.**, Shunhong, D., Koltunow, A. M. G., Randles, J. W., Beachy, R. N., and Rezaian, M. A. (2006/2007). Functional analysis of viral encoded proteins involved in the movement of *Tomato leaf curl virus*. (In preparation for Journal of Virology/Virology)

Conference presentations:

1. **Rasheed, M. S.,** Selth, L. A., Koltunow, M. A., Randles, J. W., and Rezaian, M. A. (2005). Tissue specificity of Tomato leaf curl virus and the role of V1, V2 and C4 ORFs in viral movement. 4th International Geminivirus Symposium, Cape Town, South Africa.

2. Bian X.Y., Seemanpillai M.J., **Rasheed S.M.,** Rezaian, M.A. (2004). siRNA originates from Tomato leaf curl virus coding and non-coding DNA. 4th International Geminivirus Symposium, Cape Town, South Africa,

3. Selth, L. A., Dogra, S. C., **Rasheed, M. S.,** Healy, H., Randles, J. W., and Rezaian, M. A. (2004). A novel NAC domain protein interacts with Tomato leaf curl geminivirus replication accessory protein to enhance viral replication. 4th International Geminivirus Symposium, Cape Town, South Africa.

4. **Rasheed, M. S.,** Selth, L. A., Koltunow, M. A., Randles, J. W., and Rezaian, M. A. (2004). Tissue Specificity and Movement of Tomato Leaf Curl Virus: Involvement of the V1, V2, and C4 Genes in Viral Movement. 6th Australasian Plant Virology Workshop, Gold Coast, Australia.

5. Bian, X.Y., Seemanpillai M.J., **Rasheed, M. S.,** Rezaian, M.A. (2004). Small interfering RNA originates from a geminivirus coding and non-coding DNA: evidence for silencing escape of *Tomato leaf curl virus* genes. 6th Australasian Plant Virology Workshop, Gold Coast, Australia.

6. Selth, L. A., Dogra, S. C., **Rasheed, M. S.,** Healy, H., Randles, J. W., and Rezaian, M. A. (2004). A novel NAC domain protein interacts with Tomato leaf curl geminivirus replication accessory protein to enhance viral replication. 6th Australasian Plant Virology Workshop, Gold Coast, Australia.

7. **Rasheed, M. S.,** Selth, L. A., Koltunow, M. A., Randles, J. W., and Rezaian, M. A. (2005). In situ localisation of Tomato Leaf Curl Virus DNA forms reveals cytoplasmic accumulation of ssDNA. XIII International Congress of Virology, 23rd-28th July, 2005, San Francisco, USA

8. Bian, X. Y., **Rasheed, M. S.,** Seemanpillai, M. J. and Rezaian, M. A. (2005). The role of DNA methylation in Tomato leaf curl virus escape from host silencing. XIII International Congress of Virology, 23rd-28th July, 2005, San Francisco, USA

9. Selth, L. A., Dogra, S. C., **Rasheed, M. S.,** Randles, J. W., and Rezaian, M. A. (2005). Host factors regulating replication and movement of Tomato leaf curl virus. XIII International Congress of Virology, 23rd-28th July, 2005, San Francisco, USA

10. **Rasheed, M. S.,** Bian, X. Y., Seemanpillai, M. J., and Rezaian, M. A. (2005). The role of DNA methylation in Tomato leaf curl virus escape from host silencing. 3rd Australian Virology Group Meeting, 9th-12th December, 2005, Phillip Island, Australia

11. **Rasheed, M. S.,** Selth, L. A., Koltunow, M. A., Randles, J. W., and Rezaian, M. A. (2005). Single-stranded DNA of the vascular-associated Tomato leaf curl virus accumulates in the cytoplasm: An insight into viral movement. 3rd Australian Virology Group Meeting. 9th-12th December, 2005, Phillip Island, Australia.

12. **Rasheed, M. S.,** Koltunow, M. A., Randles, J. W., and Rezaian, M. A. (2006). Functional analysis of viral encoded proteins involved in the movement of *Tomato leaf curl virus*. 8th International Congress of Plant Molecular Biology. 20th-25th August, 2006, Adelaide, Australia.

13. **Rasheed, M. S.,** Bian, X. Y., Seemanpillai, M. J., and Rezaian, M. A. (2006). The role of DNA methylation in silencing escape of *Tomato leaf curl virus*. 8th International Congress of Plant Molecular Biology. 20th-25th August, 2006, Adelaide, Australia.

14. Dogra, S. C., **Rasheed, M. S.,** Selth, L. A., Randles, J. W., and Rezaian, M. A. (2006). Interaction of Geminivirus C4 and V1 gene products with host factors having sequence homology to *Arabidopsis* shaggy-like protein kinase and UDP-glucose:protein transglucosylase. 8th International Congress of Plant Molecular Biology. 20th-25th August, 2006, Adelaide, Australia.

Chapter 1 - General introduction

1.1 – Geminiviruses

In recent years there has been tremendous progress in the area of plant virology. Most of these achievements are related to the application of molecular techniques. Plant viruses are comprised of two major groups; RNA and DNA viruses (Hull, 2002). Geminiviruses are plant DNA viruses that infect a broad variety of plant species and cause significant global crop losses (Moffat, 1999). In this chapter, geminivirus classification, genome organization, replication, transcription and functions of genes is summarised, with special emphasis on *Tomato leaf curl virus-Australia* (ToLCV-Au). For the purpose of this thesis and to keep the continuity with the previous publications from this work, the acronym TLCV is used for *Tomato leaf curl virus-Australia*. Studies describing viral localization, movement and gene silencing are reviewed.

1.1.1 *Geminiviridae*

Geminiviruses are members of the family *Geminiviridae*. The name geminivirus is coined from the word geminate (twinned) due to their unique twinned virion morphology. The viral genome consists of 1-2 small circular single stranded DNA (ssDNA) molecules that replicate through double stranded DNA (dsDNA) intermediates in the nuclei of mature infected plant cells by utilizing the host replicative machinery (Gutierrez, 2000; Hanley-Bowdoin et al., 1999).

The family *Geminiviridae* comprises four genera, distinguished by their genetic organization, host range and insect vector (Fauquet et al., 2003; Harrison & Robinson,

2002; Pringle, 1999). The genus *Mastrevirus* includes viruses with a single DNA component that infect mainly monocotyledonous plants and are transmitted by leafhoppers. Two mastreviruses, which infect dicotyledonous plants, have been identified (Boulton, 2002). Viruses of the genus *Curtovirus* are also transmitted by leafhoppers and are monopartite but infect dicotyledonous plants (Briddon & Markham, 2001). A fourth genus, *Topocuvirus*, has been established (Pringle, 1999) which contains only one virus, *Tomato pseudo curly top virus* (TPCTV). TPCTV is transmitted by a treehopper and has a monopartite genome (Briddon & Markham, 2001). Species in the genus *Begomovirus*, the largest genus of the family *Geminiviridae*, have either monopartite or bipartite genomes, are transmitted by whiteflies and infect only dicotyledonous plants (Harrison & Robinson, 2002).

1.1.2 Geminivirus DNA replication

All geminiviruses use a rolling circle replication (RCR) strategy to amplify their ssDNA genomes to produce dsDNA intermediates that serve as replicative and transcriptional templates (Heyraud et al., 1993; Saunders et al., 1991). RCR is analogous to that of many prokaryotic replicons (Baas, 1987; Novick, 1998), suggesting that the geminiviruses may have evolved from prokaryotic episomal replicons. This is supported by the observations that TLCV and other geminiviruses are able to replicate in *Agrobacterium tumefaciens* and *Escherichia coli* (Rigden et al., 1996; Selth et al., 2002).

The geminivirus replication cycle can be subdivided into two major stages in which leading and lagging strand DNA synthesis are separate events (Kornberg & Baker, 1992). The first stage involves the synthesis of a complementary-sense "minus" strand

using the single stranded virion sense "plus" strand as a template, to generate a dsDNA intermediate, which is also known as replicative form (RF). Saunders et al. (1992) has analysed the heterogenous form of complementary-sense DNA (H3 DNA) from *African cassava mosaic virus* (ACMV) infected *N. benthamiana* and suggested its role in complementary-strand replication (CSR). It is assumed that an RNA molecule is involved in the complementary-sense (minus) strand synthesis. This RNA molecule is generated either through RNA polymerase or DNA primase activity (Hanley-Bowdoin et al., 1999).

The second stage is the amplification stage of dsDNA intermediates by rolling circle replication as shown in Figure 1.1. RF serves as a template for further DNA replication and to produce plus strand DNA (Hanley-Bowdoin et al., 1999). The geminivirus plus strand DNA synthesis initiates through a site specific nick. It has been demonstrated that geminivirus replication protein is a site specific endonuclease, which cleaves virion strand viral DNA at a specific position *in vitro* (Desbiez et al., 1995). Rep protein binds to the free 5' end covalently, while the 3' end is available to prime the synthesis of plus-strand ssDNA. The subsequent events involved in plus-strand DNA synthesis are not clearly understood. However, these plus-strand ssDNA molecules are incorporated into the replication pool, transported to the adjacent cells or converted to the viral particles (Gutierrez, 1999).

Recently Jeske et al. (2001) demonstrated that geminivirus replication also involves a recombination-dependent replication (RDR) mechanism. In RDR, a viral DNA fragment recombines at a homologous site within an intact covalently closed circular DNA molecule and is extended. Replication intermediates consistent with RDR have

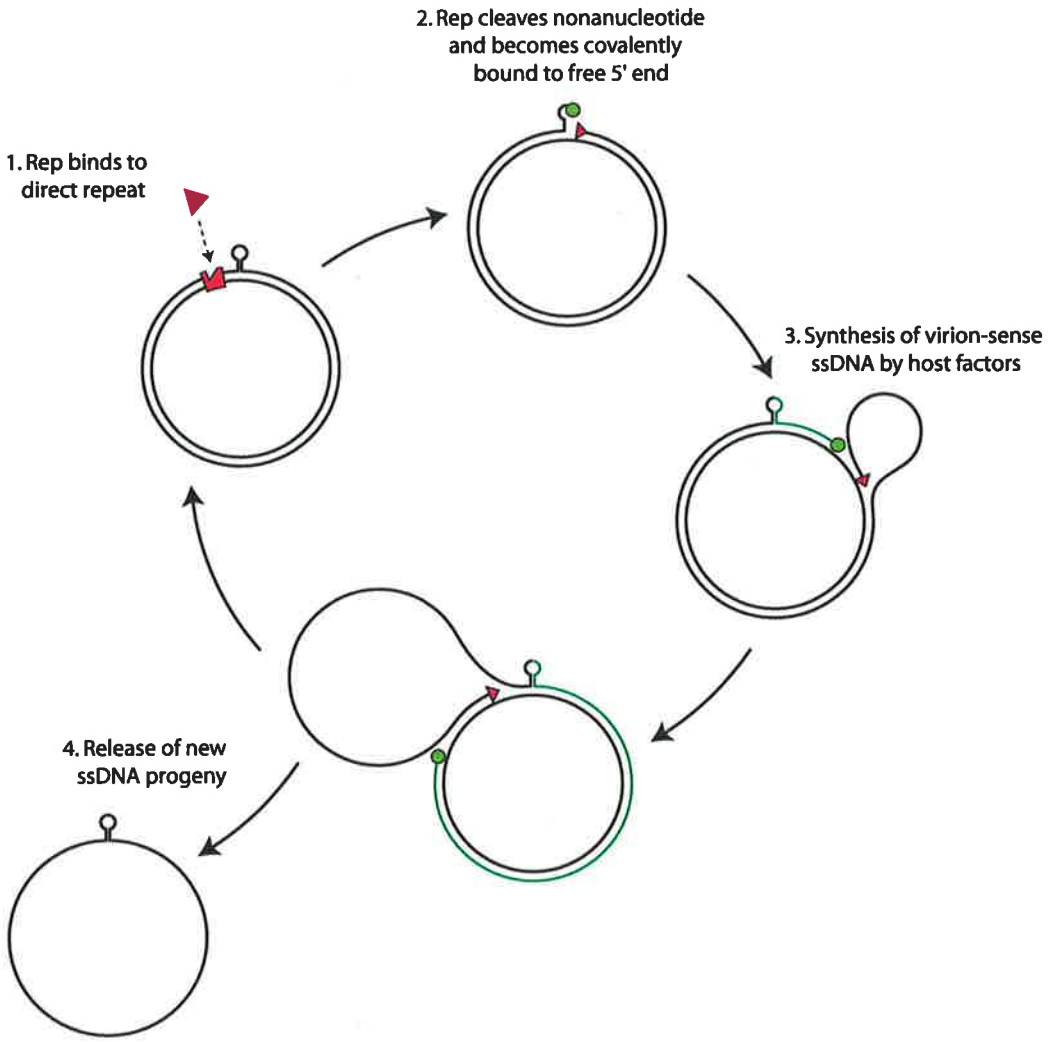


Figure 1.1. Model for geminiviral rolling-circle replication. After synthesis of complementary-sense strand to yield a double-strand, replicative form, Rep (filled red triangle) binds a direct repeat upstream of the stem-loop (1). Subsequently, the Rep protein introduces a nick into a conserved nonanucleotide sequence found in the loop region (2), and becomes covalently attached to the free 5'end. A host replication complex (filled green circle) are utilised to synthesise new virion-sense ssDNA from the 3'end (3). Termination of virion-sense replication and resolution of the genome-length ssDNAs is performed by the Rep protein (4).

been identified for ACMV, *Abutilon mosaic virus* (AbMV), *Beet curly top virus* (BCTV), *Tomato yellow leaf curl virus* (TYLCV) and *Tomato golden mosaic virus* (TGMV) (Preiss & Jeske, 2003). This mechanism allows the rescue of damaged and incomplete viral DNA and explains the production of certain replication intermediates associated with the begomoviruses. The newly produced ssDNA molecules would then be converted to dsDNA by a mechanism similar to CSR. Recent experiments in our laboratory have shown that TLCV DNA replication also involves RDR (Alberter et al., 2005).

1.1.3 Transcription of geminivirus ORFs

The genomes of geminiviruses are transcribed bidirectionally to generate mRNAs that correspond to both the virion and complementary ORFs. Transcription events occur downstream of either consensus TATA box motifs or initiator elements, suggesting that mRNAs are transcribed by host RNA polymerase II. The viral RNAs are polyadenylated and composed of multiple RNA species, indicating the complexity of geminiviral transcription (Hanley-Bowdoin et al., 1999).

1.1.4 The geminivirus origin of replication

Geminivirus DNA replication sequences for plus strand origin of replication (*ori*) have been identified and a number of significant elements are described (Fig. 1.2). The *ori* has been localized in the 5' IR, which also contains promoters for ORFs in both virion and complementary-sense strands (Lazarowitz, 1992; Saunders et al., 1991; Stenger et al., 1992). A hairpin motif in *ori* is common to all geminiviruses (Arguello-Astorga et al., 1994). In TLCV, the 2766 base sequence begins with a nonanucleotide sequence (TAATATTAC), which forms the loop of a hairpin motif (Dry et al., 1993). The

nonanucleotide sequence is also observed in the plus strand origins of other rolling circle systems (Baas, 1987). Mutations in this conserved region inhibit the viral replication *in vivo* and Rep-mediated cleavage *in vitro* (Orozco & Hanley-Bowdoin, 1996).

The binding of Rep protein to the IR is required to initiate the replication of DNA. Rep is a sequence specific DNA binding protein that interacts with sequences found upstream of the hairpin motif in the plus strand origin of TGMV (Fig. 1.2). Rep binding sites in the IRs for TLCV (Behjatnia et al., 1998), *Squash leaf curl virus* (SqLCV) (Lazarowitz et al., 1992), BCTV (Choi & Stenger, 1995) and *Wheat dwarf virus* (WDV) (Heyraud et al., 1993) have been identified. The hairpin structure does not play any role in the binding of Rep (Fontes et al., 1992). However, it has been observed that Rep binding is a species specific phenomenon. TGMV Rep that recognises the direct repeat sequence GGTAGAAGGTAG is unable to bind with the direct repeat sequence, GGAGACGGAG, of *Bean golden mosaic virus* (BGMV) (Fontes et al., 1994b). Choi and Stenger (1996) found that spacing within and between the repeats and sequence of the BCTV Rep binding sites are also crucial for species specific recognition. However, tight specificity for an interaction between Rep and the plus strand origin is not required in the case of the satellite DNA associated with TLCV (Dry et al., 1997). Recently it has been observed that mutants of TLCV as well as sat-DNA having mutations in the direct repeat retained their ability to replicate in tomato (Lin et al., 2003). The reason for binding of Rep to direct repeats is not clear; however the presence of these motifs in most of the geminiviruses is crucial for replication. It has been observed that mutations in the direct repeat impair Rep/DNA binding *in vitro* as well as interfere with viral replication *in vivo* (Fontes et al., 1994a; Orozco & Hanley-Bowdoin, 1998). In

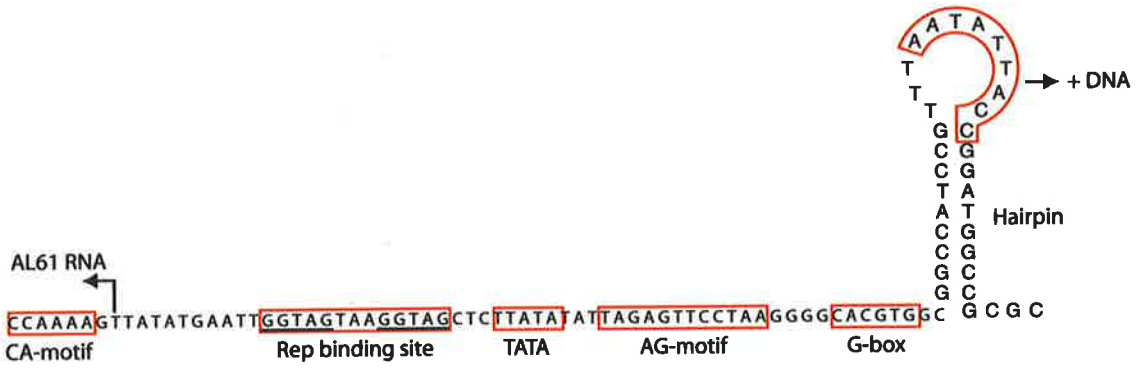


Figure 1.2. The TGMV plus-strand origin of replication and C1 (Rep) promoter. The DNA sequence corresponding to TGMV A positions 54-153 is shown. Only the top strand of the duplex DNA is given. The initiation sites and direction of synthesis for plus-strand DNA replication and AL61 transcription are indicated. Other functional elements are boxed. The hairpin structure is drawn and the conserved nonanucleotide loop sequence is marked (reproduced from Hanley-Bowdoin et al., 1999).

begomoviruses, positive-strand origin of replication overlaps the complementary sense promoter. The TATA box is located 3 bp upstream of the Rep binding site, and the G-box is positioned at the base of the hairpin structure that is required for the transcription of *C1* and *C4* ORFs (Eagle & Hanley-Bowdoin, 1997).

Two additional elements have been identified in the TGMV *ori*, an AG-motif and a CA-motif (Fig 1.2). The AG-motif is essential for origin function and is located in between the hairpin and the Rep binding site (Orozco et al., 1998) whereas the CA-motif acts as an efficiency element and is present outside of the minimal origin immediately upstream of the Rep binding site. These elements are thought to bind with the host factors and thus facilitate the initiation of plus-strand DNA synthesis (Hanley-Bowdoin et al., 1999).

1.1.5 Genome organization of begomoviruses

Begomoviruses have complex viral genome and transcription of genes for viral proteins occurs from the circular double stranded DNA (dsDNA) intermediates. The transcriptionally active forms of DNA, dsDNA, have two strands containing six to seven genes, whose transcription occurs in complementary sense (c-sense) and virion sense (v-sense) directions, which are separated by the intergenic region (IR). The intergenic region includes a stem-loop structure, with the loop having nonanucleotide TAATATTAC sequence, which is conserved among all geminiviruses (Hanley-Bowdoin et al., 1999). Proteins encoded by these genes are involved in viral DNA replication, regulation of viral transcription, intra- and intercellular movement, encapsidation, insect vector transmission and disease symptoms in the host plant.

1.1.6 Tomato leaf curl virus (TLCV)

Tomato leaf curl (TLC) and tomato yellow leaf curl (TYLC) are severe viral diseases of cultivated tomato (*Solanum lycopersicum* L.) in the tomato growing regions of the world. These viral diseases result in a significant reduction in the yield of tomato crop around the world, including Australia (Behjatnia et al., 1996), India (Saikia & Muniyappa, 1989), Middle East, Mediterranean, South East Asia, Africa and America (Nakhla & Maxwell, 1998). Phenotypic symptoms of both diseases in the infected plants include curling of leaves, chlorosis or yellowing of leaves, stunted plant growth and low or no fruit set (Nakhla & Maxwell, 1998). Several begomoviruses are the causal agent of these diseases and losses to tomato production range from 28-92%, depending on the suitability of conditions for viral infection (Nakhla & Maxwell, 1998).

The first report of TLC disease in Australia was given by Aldric in 1970 in the Northern Territory (Behjatnia et al., 1996). The disease is now spreading in the Far Northern Queensland. The causal agent, TLCV, is a monopartite *begomovirus*. A large population of B biotype of *Bemisia tabaci*, which is a very efficient vector of TLCV, is present in Queensland and Northern New South Wales (Fig 1.3) including the major tomato-growing regions in Australia. There is a small pocket in Far Northern Queensland where TLCV and B biotype of *B. tabaci* overlap. Therefore, it is thought that a TLCV epidemic may spread into this major tomato-growing region (M. A. Rezaian, personal communication). A satellite DNA (sat-DNA) (Dry et al., 1997) and sub-genomic viral DNA components (Behjatnia, 1997) are associated with TLCV and depend on TLCV for their replication. Studies related to the host pathogen interactions, viral localization and movement are required to design effective control measures against this particular disease.

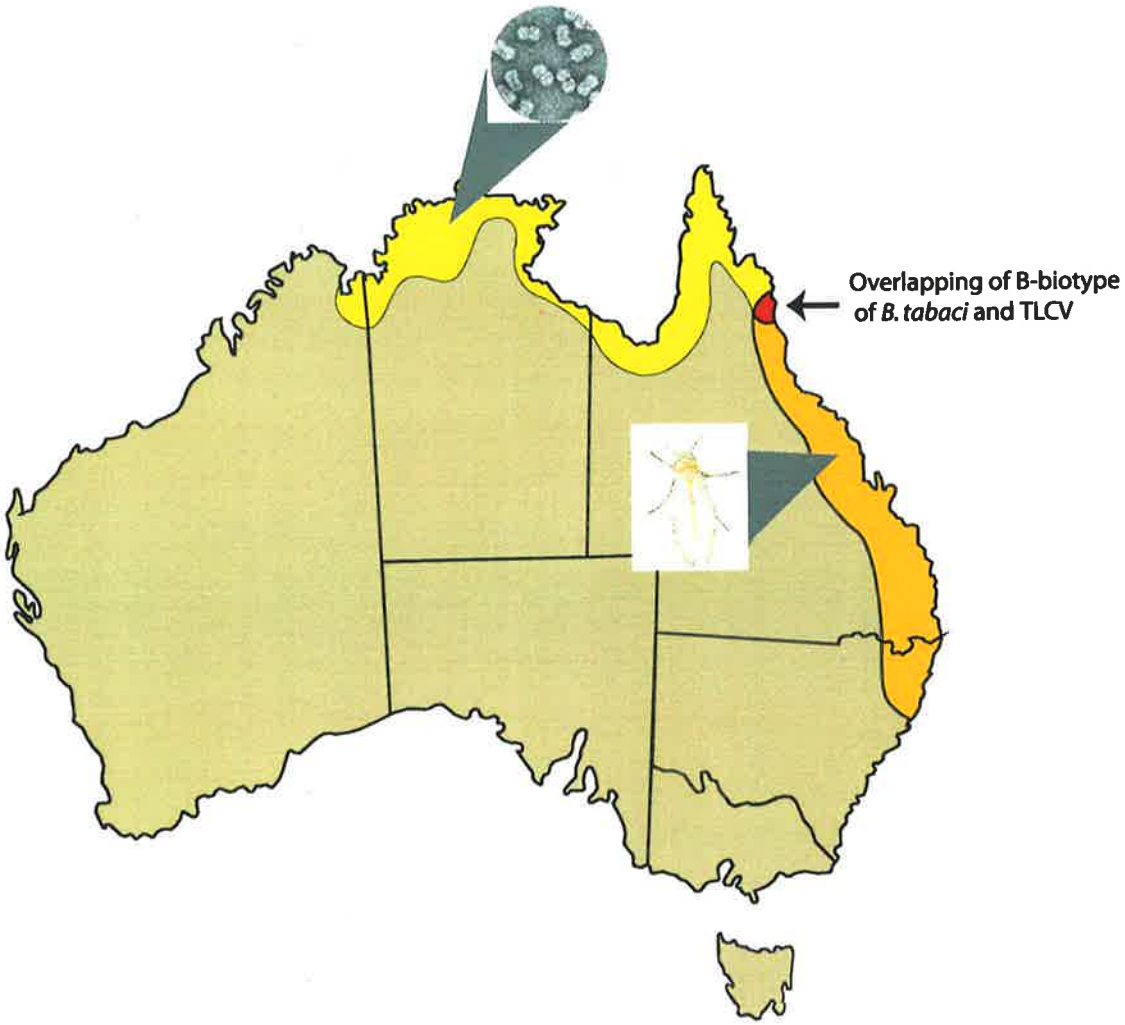


Figure 1.3. The distribution of TLCV and B-biotype of *B. tabaci* in Australia (reproduced from Stonor et al., 2002).

1.1.6.1 Genome organization of TLCV

The genome organization of TLCV resembles other dicotyledonous plant-infecting monopartite begomoviruses (Dry et al., 1993). TLCV contains a circular, ssDNA genome of 2766 nucleotides that encodes six open reading frames (ORFs) (Dry et al., 1993). Two open reading frames (*V1* and *V2*) are encoded in the virion sense while four (*C1*, *C2*, *C3* and *C4*) are encoded on the complementary sense DNA (Fig. 1.4).

1.1.6.2 Transcription of the TLCV ORFs

TLCV-encoded transcripts from infected tomato have been characterized (Mullineaux et al., 1993). Four major virus-specific transcripts were identified by nuclease protection assays and by the technique, rapid amplification of cDNA ends polymerase chain reaction (RACE-PCR), using TLCV-specific primers. The complementary-sense strand generates two RNA species; one spans the *C1* (and *C4*), *C2* and *C3* ORFs while a second internal RNA covers *C2* and *C3*. Two transcripts are produced by the virion-sense strand and are situated on either side of the start codon of *V1* ORF.

1.1.6.3 Functions of the TLCV genes

The functions of genes of the geminiviruses have been studied extensively, particularly for the bipartite begomoviruses like TGMV. In contrast, the functions of TLCV ORFs have not been well characterized. Therefore, in this section, gene functions of the geminiviruses will be considered with an emphasis on bipartite begomoviruses, and where applicable, on monopartite begomoviruses including TYLCV and TLCV.

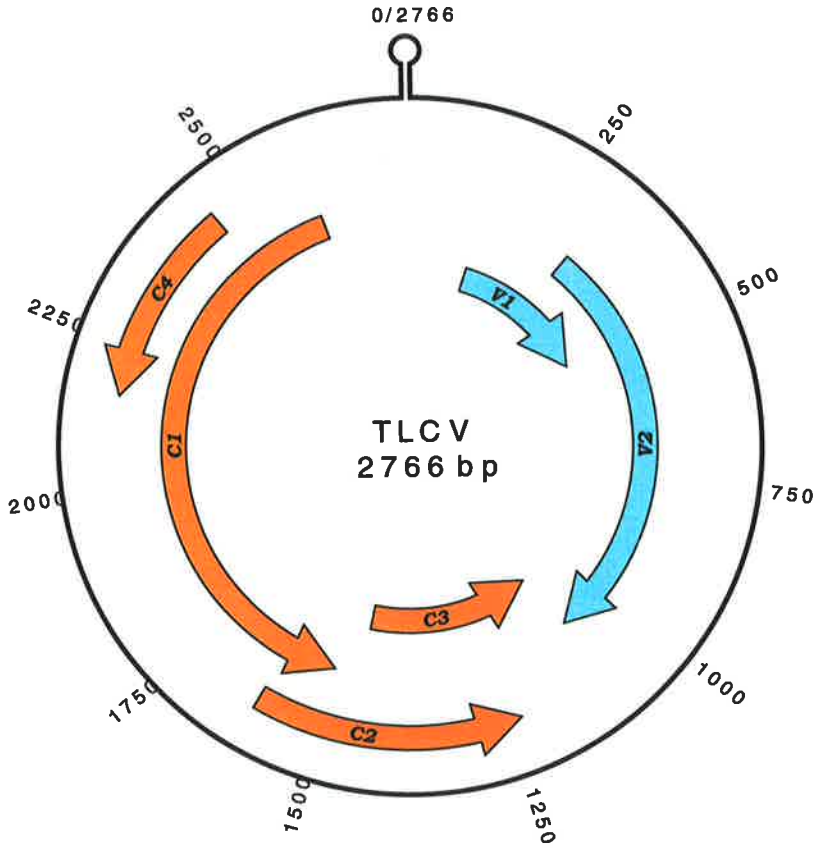


Figure 1.4. Genome organization of TLCV. ORFs on the virion-sense (clockwise) strand and the complementary-sense (anticlockwise) strand are shown by arrows. The position of the conserved stem-loop structure is marked (reproduced from Dry et al., 1993).

1.1.6.3.1 *V1*

The *V1* ORF of begomoviruses has been implicated in symptom development and ssDNA accumulation (Rigden et al., 1993; Wartig et al., 1997). It has been observed that V1 is involved in cell-to-cell spread of *Maize streak virus* (MSV) (Lazarowitz et al., 1989). However, it is thought that this particular ORF is not important for the systemic spread of virus in the plant. Rigden et al. (1993) showed that disruption in the *V1* ORF did not affect the viral dsDNA synthesis but produced symptomless systemic infection with a reduced titre of all forms of viral DNA in inoculated plants. In contrast to this result Wartig et al. (1997) reported that disruption of the *V1* ORF of *Tomato yellow leaf curl Sardinia virus* (TYLCSV) abolished systemic infection in tomato plants and thus was required for successful infection in the host. It has been observed that V1 of TYLCV enhanced the capacity of coat protein (CP) to mediate nuclear export of DNA, possibly via an interaction with a CP-DNA complex (Rojas et al., 2001). Rojas et al. (2001) also suggested that V1 is involved in the cell-to-cell movement of viral nucleic acid, possibly by an interaction with endoplasmic reticulum (ER) as V1 proteins co-localized with the ER. Experiments to detect the subcellular localization of TLCV V1 protein have not been done.

1.1.6.3.2 *V2*

The V2 gene of the monopartite begomoviruses encodes the viral coat protein, which is common in other monopartite geminiviruses (Boulton et al., 1993; Dry et al., 1993; Morris-Krsinich et al., 1985; Rigden et al., 1993). The coat protein is required for encapsidation to protect the viral genome. Long distance systemic movement of virus may also require a functional coat protein (Hull, 2002). It has been observed that mutations in the TLCV V2 coat protein gene disrupted spread of the virus (Rigden et

al., 1993) while none of the mutations in the *V2* ORF of the monopartite TYLCV prevented the synthesis of CP (Wartig et al., 1997). Capsid protein of monopartite TYLCV and bipartite SqLCV has the ability to interact with ssDNA and is involved in the accumulation of viral ssDNA genomes (Palanichelvam et al., 1998; Qin et al., 1998). There is no report about the DNA binding ability of TLCV V2 protein. Rojas et al. (2001) has reported that TYLCV CP accumulates in the nucleus and is involved in the nuclear import and export of viral DNA. So far no research has been done to determine whether TLCV CP is a nuclear shuttle protein.

1.1.6.3.3 *C1*

The protein encoded by the TLCV *C1* ORF is designated as a replication-associated protein (Rep), and is the only viral protein essential for viral DNA replication (Behjatnia et al., 1998). Rep localizes to the nuclei of infected plant cells (Nagar et al., 1995) and plays an important role in geminivirus replication and transcription (Laufs et al., 1995a). Rep has various different functions, which are summarised in Figure 1.5 and some of them are described below.

1.1.6.3.3.1 DNA binding, nicking and ligation activities

Studies with TLCV (Behjatnia et al., 1998), TYLCV (Jupin et al., 1994), TGMV (Thommes et al., 1993) and other geminiviruses have shown that Rep confers virus specific recognition and binds to the direct-repeat sequences in their respective *ori* (as explained in section 1.1.4).

Rep introduces a nick in the plus strand between the 7th and 8th conserved nucleotide in the nonamer motif, ^{1}TAATATT$^{\downarrow 8}$AC, (Laufs et al., 1995b). The newly generated 5' end

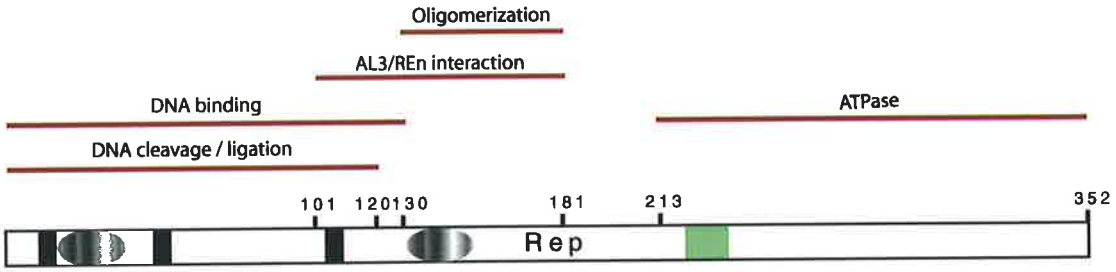


Figure 1.5. TGMV Rep domains. Solid boxes mark the locations of the three conserved DNA cleavage motifs in the Rep protein. The green box shows the location of the ATP binding motif. The shaded circles indicate predicted sets of α helices. Solid brown lines above the protein mark the location of the functional domains for oligomerization, AL3/REn interaction, DNA binding, DNA cleavage/ligation and ATPase activity. The numbers correspond to amino acid positions in Rep (reproduced from Hanley-Bowdoin et al., 1999)

of the cleaved strand becomes covalently linked to the Rep, while the 3' hydroxyl nucleotide at position 7 becomes available for plus strand DNA synthesis (Laufs et al., 1995b). Upon the completion of complete replication, the newly synthesized strand is cleaved by Rep at the same position and 3' hydroxyl is joined to the 5' phosphate of the strand to release a circular ssDNA molecule of the same length as the genome (see Fig. 1.1) (Laufs et al., 1995b).

1.1.6.3.3.2 NTPase activity

It has been reported that Rep proteins of all geminiviruses have a sequence comparable to the consensus nucleotide triphosphate-binding (NTP) motif and exhibit a weak similarity to DNA helicase (Gorbalenya & Koonin, 1989). ATPase activity has been identified for TGMV and TYLCV Rep (Desbiez et al., 1995; Orozco et al., 1997), whereas there is no report available to date for helicase activity in geminivirus replication protein. Moreover it has been observed that the DNA independent nature of the ATPase activity is not consistent with Rep as a DNA helicase (Hanley-Bowdoin et al., 1999). It is possible that ATP hydrolysis mediates a conformational change that is required for the initiation of plus strand synthesis (Hanley-Bowdoin et al., 1999). This is supported by the observation that the mutation in the NTP binding motif prevents Rep from supporting viral replication but has no effect on Rep-mediated transcriptional repression (Desbiez et al., 1995; Eagle et al., 1994) and the specific Rep and DNA interactions required for transcriptional repression are a subset of those required for DNA replication (Gladfelter et al., 1997).

1.1.6.3.3.3 Transcriptional regulation

It has been observed that Rep from ACMV (Hong & Stanley, 1995) and TGMV (Hanley-Bowdoin et al., 1999) regulates its own expression during transcription. This regulatory activity might be due to the binding of Rep to the direct repeat in *ori* (see Section 1.1.4). It is thought that binding of Rep could interfere with the assembly of transcriptional machinery, as the Rep binding site is located between the TATA box and the *rep* transcriptional start point (Hong & Stanley, 1995). It has been observed that the regulatory activity of Rep is located in the region where its DNA binding domain is present (Hong & Stanley, 1995).

1.1.6.3.3.4 Induction of host replication machinery

Geminiviruses rely on host replication machinery and have the ability to replicate in differentiated cells, which generally do not contain detectable levels of replication enzymes (Nagar et al., 1995). It has been shown that geminiviruses have the ability to modify the host cell replication machinery and thereby induce the replication of host DNA (Nagar et al., 2002). This resembles mammalian DNA viruses, which use a combination of mechanisms to induce replication protein in their hosts (Labrie et al., 1995; Lee et al., 1991). Recent work with plant viruses suggests that Rep is required for activating a replication-permissive state by interacting with plant retinoblastoma (RB) related protein, which is involved in the regulation of the plant cell cycle.

1.1.6.3.4 *C2*

The product of the *C2* ORF of monopartite begomoviruses is required for systemic infection and is a transcriptional activator protein (TrAP). It has been observed that a *C2* mutant of TLCV exhibited the capability of autonomous replication in tobacco leaf

strips (Dry et al., 1997). The mutants of the *C2* gene product resulted in the reduction of ssDNA levels and an increase in the levels of dsDNA replicative forms. This suggests that the *C2* gene product of TLCV transactivates the expression of virion sense genes (Dry et al., 1997). The protein encoded by *C2* ORF of TYLCV-Sr is also required to establish infection in tomato but not in *Nicotiana benthamiana* (Wartig et al., 1997). In contrast the *C2* ORF of BCTV is not required for infectivity (Hormuzdi & Bisaro, 1995).

In bipartite geminiviruses, the AC2 gene product seems to be involved in viral movement. Sunter and Bisaro (1991) reported that the AC2 protein of TGMV is a transactivator of coat protein and movement protein genes. They also observed a reduced level of ssDNA in transient assays of mutated *AC2* ORF and suggested that mutated *AC2* prevents systemic viral movement in plants. Similarly AC2 has also been reported to regulate the expression of *BC1* in ACMV (Haley et al., 1992).

There is an increasing debate about defence mechanisms of plants against viruses, involving post-transcriptional gene silencing (PTGS). Recent findings suggest that the C2/AC2 protein of geminiviruses may have some role in gene silencing. The protein encoded by *AC2* gene of ACMV is thought to have a role in gene silencing suppression (Voinnet et al., 1999). Similarly, transgenic plants expressing the TGMV *AL2* gene and BCTV *L2* (*C2*) gene exhibit enhanced susceptibility to infection by other DNA and RNA viruses and suggest these gene products have the ability to suppress a host defence response to a range of viruses (Sunter et al., 2001). Furthermore, the role of TLCV C2 protein as a suppressor of PTGS has also been verified in our laboratory (Selth et al., 2004).

1.1.6.3.5 *C3*

The role of the *C3* ORF in TLCV is not known (Dry et al., 2000). However C3/AL3 proteins significantly enhance viral DNA accumulation in both monopartite and bipartite geminiviruses through an unknown mechanism (Elmer et al., 1988). It has been reported that AL3 is localized to the nuclei of infected plant cells (Nagar et al., 1995; Pedersen & Hanley-Bowdoin, 1994). These findings suggest that AL3 might act with AL1 during initiation of viral DNA replication. The gene product of begomovirus AC3 is not required for viral replication; while it seems to affect viral DNA synthesis efficiency by enhancing the levels of viral replication (Sunter et al., 1990).

It has been demonstrated that TGMV AL3 interacts with a maize RB homologue and suggested that it also plays a role in modifying plant cells, to create a replication supportive environment (Settlage et al., 2001). Experiments in this laboratory have verified that the product of *C3* gene of TLCV interacts with a tomato protein of the NAC family named SlNAC1 and enhances viral replication (Selth et al., 2005).

1.1.6.3.6 *C4*

The protein encoded by the *C4* gene of TLCV plays different roles in virus movement and symptom development. It has been suggested that the *C4* gene of TYLCV is required for systemic viral movement (Jupin et al., 1994). On the other hand, Rigden et al. (1994) have suggested that the *C4* gene of TLCV encodes a protein that is not required for TLCV replication and systemic spread, but is involved in symptom severity. Similarly, Krake et al. (1998) have reported that the *C4* gene from TLCV developed severe disease symptoms in transgenic plants. Similar results of disease development were reported by Stanley and Latham (1992) for BCTV *C4* ORF. It has

been demonstrated that transgenic plants of *N. benthamiana* which ectopically express BCTV *C4* develop abnormally and produce tumours (Latham et al., 1997). These results suggest that *C4* can cause cell division in plants in the absence of viral proteins possibly with the interaction of host factors (Hanley-Bowdoin et al., 1999). Recently Vanitharani et al. (2004) reported that AC4 proteins (the bipartite homologue of C4) from ACMV (Cameroon strain) and Sri Lankan cassava mosaic virus are suppressors of PTGS. Therefore, there is a need to explore different functions of C4 regarding its role in viral movement, cell division and suppression of gene silencing.

1.1.7 Satellite and sub-genomic DNAs associated with TLCV

RNA virus infected plants may contain subgenomic RNA and subviral agents, such as viroids and satellites (Hull, 2002). Satellites can be differentiated from subgenomic nucleic acids by their lack of appreciable sequence homology to the genome of helper virus. Virus-specific DNA satellites are also associated with a range of geminiviruses including ACMV, BCTV, TGMV, TYLCV and WDV (Czosnek et al., 1989; Dry et al., 1997; Frischmuth & Stanley, 1992; Hayes et al., 1988; MacDonald et al., 1988; Stanley & Townsend, 1985). Dry et al. (1997) reported a satellite DNA (sat-DNA) associated with TLCV, which was the first report of a DNA satellite. This sat-DNA is encapsidated by TLCV CP and is dependent on helper virus for its replication (Dry et al., 1997). Sub-genomic (SG) DNA molecules of different size were observed in TLCV infected plants (Behjatnia, 1997). SG DNAs are encapsidated by TLCV CP and replicate in the presence of TLCV. These SG DNAs interfered with virus replication and were classified as defective-interfering DNAs, however the exact role of SG DNAs in the geminivirus multiplication is not clear (Behjatnia, 1997).

Another type of DNA component identified in cotton plants infected with *Cotton leaf curl Multan virus* (CLCuMV) was almost half the size of the genome of its helper begomovirus. This DNA molecule encodes a Rep-like protein which resembles that of the nanoviruses and was named DNA-1 (Mansoor et al., 2003; Mansoor et al., 1999; Saunders & Stanley, 1999). DNA-1 can replicate by itself, but requires the helper virus for its movement and insect transmission (Saunders & Stanley, 1999). DNA-1 cannot strictly be defined as a sat-DNA because of its autonomous replication (Mayo et al., 2005). It has been suggested that DNA-1 molecules could be derived from either nanovirus like components or begomovirus-adapted nanovirus components (Briddon & Stanley, 2006).

The most important satellite component identified so far is DNA β, which was first characterized in association with *Ageratum yellow vein virus* (AYVV) (Saunders et al., 2000). This satellite molecule was designated as DNA β because in some respects it functionally resembled the DNA-B molecule of bipartite begomoviruses (Saunders et al., 2000). Since then such satellite molecules have been identified in a wide variety of plant species associated with a range of monopartite begomoviruses throughout Africa and Asia (Mansoor et al., 2006). DNA β requires a helper virus for replication, encapsidation, movement and insect transmission and is therefore designated as a satellite molecule (Mayo et al., 2005). These sat-DNAs are half the size of the helper virus genome and have no sequence homology with the helper virus. Most importantly, these satellite molecules modulate symptom development (Saeed et al., 2005; Saunders et al., 2004).

DNA β has three characteristic features: (i) a single, highly conserved ORF present on the complementary-sense strand, known as βC1, (ii) a conserved sequence of approximately 200 nucleotides recognized as the satellite-conserved region (SCR) and (iii) an adenine (A)-rich region. The exact function of the A-rich region is currently unknown. However, the deletion of the A-rich region does not affect the replication, encapsidation and symptom development capabilities of the molecule (Tao et al., 2004). Mutagenesis studies of the DNA β*C1* ORF and stable expression of this gene in transgenic plants have shown that βC1 is a determinant of pathogenicity (Saeed et al., 2005; Saunders et al., 2004). It has been suggested that gene silencing suppressors induce pathogenic effects in plants (Voinnet et al., 1999). A recent report demonstrating the role of βC1 as a suppressor of gene silencing (Cui et al., 2005) supports this argument. However, the mechanism(s) by which βC1 induces symptoms remains unclear.

Satellite DNA β molecules have been identified only recently and have produced diverse monopartite begomovirus-DNA β disease complexes. There is a need to investigate the association of this sat-DNA with the helper virus at the cellular level and to determine its precise role in disease development.

1.2 Tissue Tropism

Plant viruses infect different cells and tissues to establish a systemic infection in the host. Localization of a particular virus to the specific cells and tissues is termed a tissue tropism. Different viral and host factors determine tissue specificity. Factors responsible for tissue tropism in animal viruses have been extensively studied, whereas identification of the determinants of plant viral localization has not yet been achieved.

In this section the literature review focuses mainly on tissue tropism, and possible factors which determine the localization of geminiviruses in host cells and tissues.

1.2.1 Viral localization and phloem specificity

Most plant viruses are restricted to certain tissues during infection. Some of the viruses are phloem restricted and are found usually in phloem parenchyma, companion cells and sieve elements, whereas others have the ability to invade mesophyll. Similarly, a few viruses appear to be limited to the phloem during early stages of infection but at later stages they are found in mesophyll as well. Viruses also exhibit uneven distribution in leaves to show mosaic patterns (Hull, 2002). Molecular mechanisms that determine the tissue specificity and distribution of viruses are currently unknown. It is also possible that certain host factors responsible for viral replication and movement are not available outside the vascular system.

Different geminiviruses exhibit different patterns of tissue specificity; SqLCV (Ward et al., 1997), BGMV (Kim et al., 1978), BCTV (Latham et al., 1997) and AbMV (Horns & Jeske, 1991) have been detected only in phloem tissues. On the other hand TGMV has been observed in most cell types of leaf, including mesophyll cells, in the model host *Nicotiana benthamiana* (Nagar et al., 1995). It has been reported that *Euphorbia mosaic virus* (EuMV) is confined to phloem in its natural host *Euphorbia heterophylla* whereas it has the ability to invade mesophyll cells in he experimental host *Datura stramonium* (Kim & Lee, 1992), suggesting that the host-pathogen interaction regulates the localization of viruses.

As indicated above, TGMV and BGMV exhibit differential tissue tropisms in the host. It has been observed in co-inoculation studies that TGMV has the ability to overcome the phloem limitation of BGMV (see Figure 7) (Morra & Petty, 2000). In subsequent experiments, a hybrid virus, in which the fragments of BGMV genome were replaced with the analogous genes of TGMV, was used to determine the viral factors responsible for mesophyll-invasiveness of BGMV in co-inoculation studies. It was found that BRi (the non-coding region upstream from the TGMV *BR1* ORF) is required along with *AL2/AL3* ORFs or *BL1/BR1* ORFs for mesophyll invasion of hybrid virus (Morra & Petty, 2000).

Recent experiments on in situ hybridization with different bipartite geminiviruses including AbMV (Saunders et al., 2001b), *Cabbage leaf curl virus* (CabLCV) (Qin & Petty, 2001) TGMV and BGMV (Morra & Petty, 2000) indicate that there is an accumulation of viral DNA in nuclei of host cells (Fig. 1.6 and 1.7). There is no conclusive report describing the localization of different forms of begomovirus nucleic acid in host cells.

1.2.2 Possible factors for tissue tropism

The factors that can direct the exit of a given virus from the vascular system have not been studied in detail. However the possible factors which determine the phloem-limitation or mesophyll-invasion of a particular virus are described below.

1.2.2.1 Mechanical or non-mechanical transmission

It is generally considered that viruses, which are not mechanically transmissible, are phloem-limited whereas viruses, which are mechanically transmissible, are mesophyll-

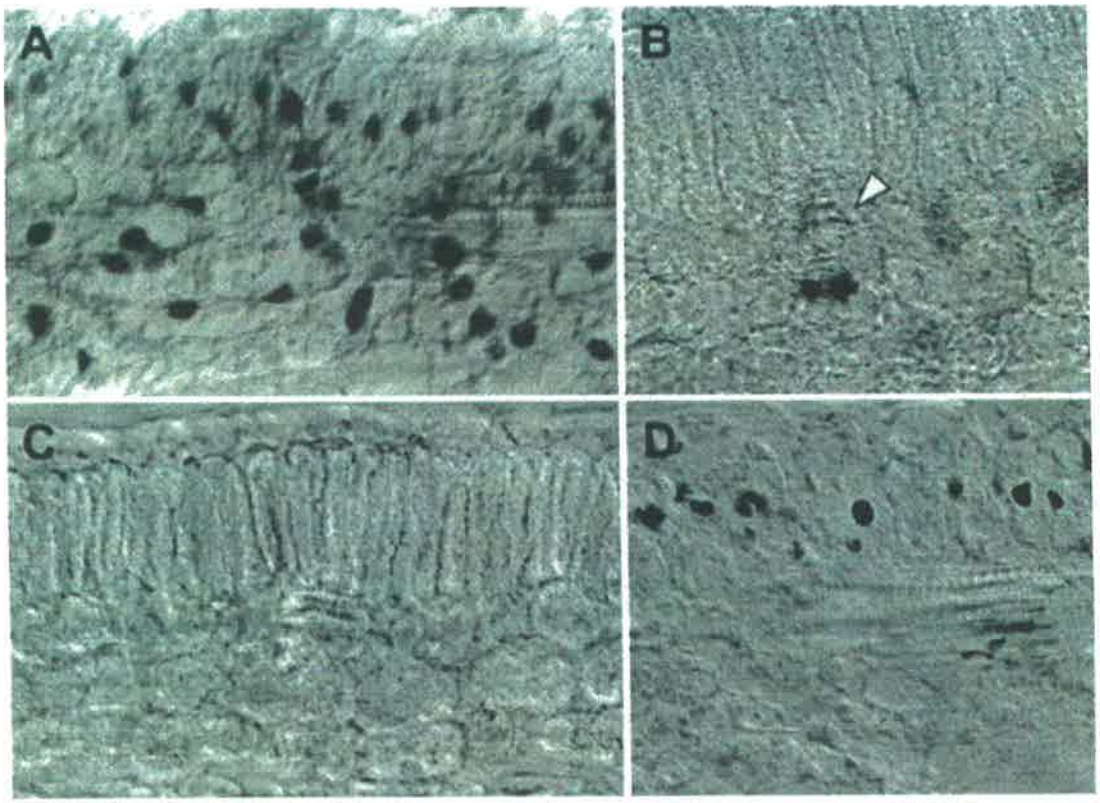


Figure 1.6. In situ localization of wild-type begomoviruses in single and double infections (x400 magnifications) (Morra et al., 2000)

(A) Sections from a plant infected with wild-type TGMV and hybridized with the TGMV-specific probes. Hybridization signals were detected in nuclei of spongy and palisade mesophyll cells as well as in those of vascular-associated cells. **(B)** Sections from a plant infected with wild type BGMV and hybridized with BGMV-specific probes. Hybridization signals were detected in nuclei of vascular-associated cells. A white arrowhead indicates the location of a vascular bundle passing perpendicular to the plane of the section. **(C)** Sections from an uninfected plant incubated with the TGMV-specific probes. No non-specific hybridization was detected. **(D)** Sections from a plant infected with both wild-type BGMV and wild-type TGMV and hybridized with the BGMV-specific probes. The presence of hybridization signals in nuclei of palisade mesophyll cells indicates that BGMV can infect these cells following double infection with TGMV.

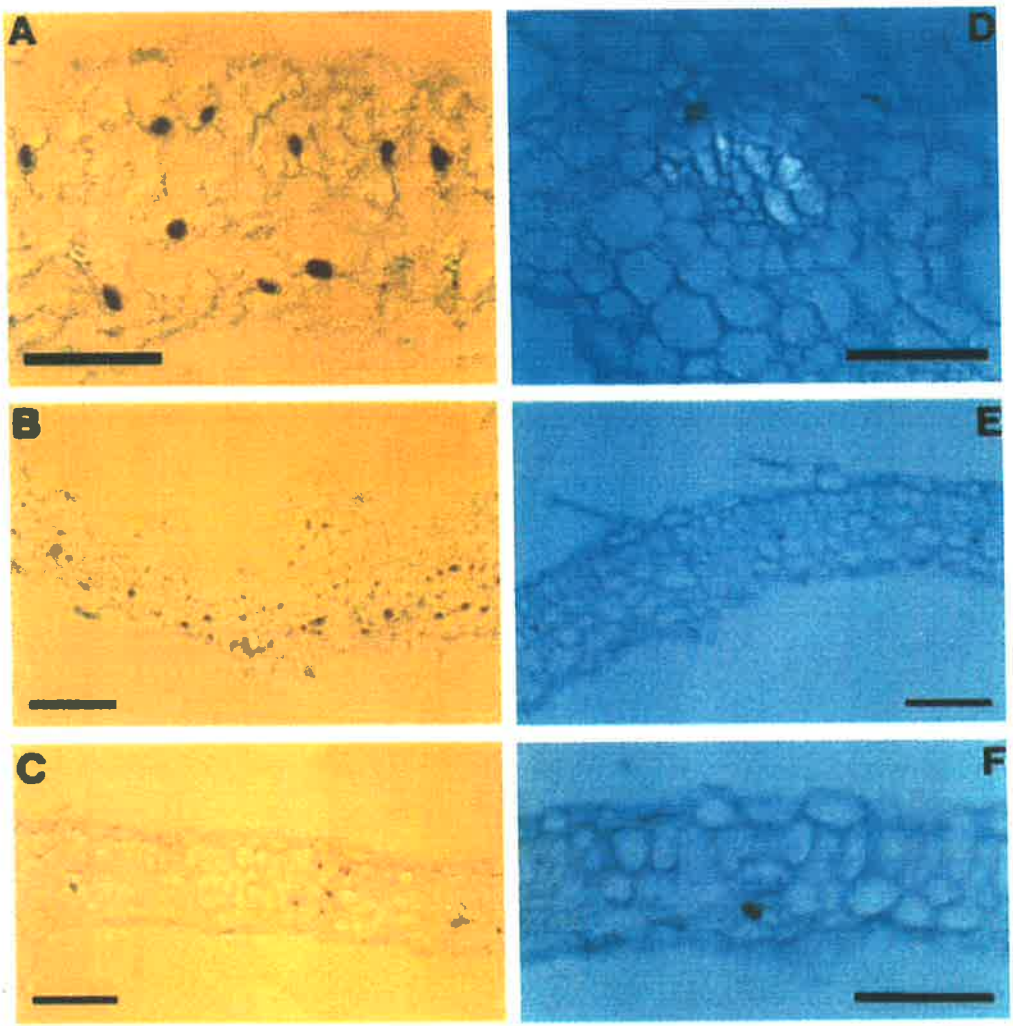


Figure 1.7. Comparison of TGMV and AbMV DNA distribution in sections of systemically infected *N. benthamiana* leaves 17-18 days post inoculation (dpi). (Wege et al., 2001)

Tissues were taken from plants infected with TGMV **(A-C)** and AbMV **(D-F)**. Nuclei containing viral DNA appear darkly stained. TGMV DNA is detectable in the nuclei of spongy and palisade parenchyma cells at a late stage of infection when the leaves exhibit severe leaf curl symptoms (A, B). Earlier in infection, TGMV DNA is predominantly associated with phloem cells and, to a lesser extent, adjacent palisade parenchyma cells (C). AbMV DNA is confined to the nucleus of a phloem cell of a large leaf vessel (D). Even at a later stage of infection, AbMV DNA is confined to nuclei of cells adjacent to xylem elements in the vascular bundles (E), also shown at higher magnification (F). Sections were examined using differential interference contrast (DIC) microscopy and Astrablue counterstain. Bars = 50 μm.

invasive. Wege et al. (2001) has shown that mechanically transmissible geminivirus *Sida micrantha mosaic virus* (SimMV) closely related to AbMV, is phloem-limited in systemically infected plants suggesting that mechanical transmission is not a necessary norm for mesophyll invasion. Most of the insect vectors of persistently transmitted viruses transfer viruses into the sieve tubes via their stylet (Hull, 2002). Phloem-limited viruses are unable to move out of the vascular system. It has been reported that virus exit from the vascular bundles in a leaf may only be possible before the sink-source transition (Roberts et al., 1997). However, how and where viruses exit the phloem is unknown.

1.2.2.2 Host responses

Cross protection was recognized in the beginning of the 20th century in crop plants (Hull, 2002). The mechanism of cross protection has remained a mystery, as plants do not have an immune system. However this has been recently explained through an understanding of gene silencing events and the action of suppressors of gene silencing. It seems that gene silencing activates the plant's adaptive defence mechanism against viruses (Waterhouse et al., 2001). Barker et al. (2001) have suggested that an RNA-mediated form of resistance which is analogous to post transcriptional gene silencing operates in non-vascular cells and may be part of the mechanism that restricts *Potato leaf roll virus* (PLRV) to vascular tissues in naturally infected plants. Voinnet et al. (1999) have also proposed the same mechanism for phloem limitation based on putative tissue-specific host defence responses.

Geminivirus replication, except for viral replication associated protein, depends on host proteins. Most cells in mature plants have completed the cell cycle and have undergone

differentiation and do not support active DNA replication (Nagar et al., 1995). To overcome this problem, geminiviruses direct their hosts to create a replication-supportive environment (Egelkrout et al., 2001; Nagar et al., 1995). Some geminiviruses are phloem restricted (Sanderfoot & Lazarowitz, 1996) and may replicate in vascular parenchyma cells which contain replicative components like the cyclin-dependent kinase cdc2a (Martinez et al., 1992). These results suggest that host responses determine the tissue specificity of viruses in host plants.

1.2.2.3 Viral encoded movement proteins

The ability to invade the different tissues of a host plant is an important aspect of viral movement and functions. Another possible explanation of phloem restriction is that the movement proteins encoded by virus cannot function in mesophyll cells, or are unable to mediate the initial escape of virus from vascular system to mesophyll (Qin & Petty, 2001). However, this possibility has yet to be verified experimentally

1.2.2.4 Other factors

There might be a variety of mechanisms which are responsible for virus tissue tropism, but in some cases localization of plant viruses can be affected by environmental conditions (Ding et al., 1999b), or the developmental stage of the host (Wang et al., 1996).

There is a need to explore different molecular mechanisms, which govern the localization of plant viruses to their host cells, particularly monopartite begomoviruses, as there is no report available which can explain the tissue specificity of TLCV.

1.3 Viral Movement

Plant viruses must have the ability to overcome various transport barriers to cause an infection in the host. Viral encoded proteins are the principal elements by which all plant viruses counter these transport barriers. The movement of plant viruses generally occurs in two distinct phases to establish systemic infection in the host: cell-to-cell (local) movement and long distance (systemic) movement. During the recent years, molecular techniques, including plant transformation, infectious clones, recombinant protein systems, microinjection and reporter proteins have played a significant role in understanding virus movement particularly cell-to-cell movement. Different viral proteins are required for these two types of movements. Plant viruses encode movement proteins (MPs) that are involved in the cell-to-cell transport of the virus (Lucas & Gilbertson, 1994). It has been observed that CP is required for long distance movement in the majority of viruses, but it is unclear whether CP is required for virions or as a nonvirion nucleoprotein complex (Gilbertson & Lucas, 1996). A proposed model for intra- and inter-cellular movement of geminiviruses is presented in Figure 1.8, which describes the role of different viral encoded proteins for viral trafficking. In addition to the role of viral encoded proteins, plant viruses may also rely on various host factors for their movement, including host proteins and cytoskeleton elements. Animal viruses have been shown to exploit various components of the cytoskeleton for intracellular transport. Recent findings suggest that microtubules are involved in the intracellular movement of *Tobacco mosaic virus* (TMV) and its targeting to the plasmodesmata (Pd) (Boyko et al., 2000; Kotlizky et al., 2001). No conclusive data are yet available to confirm the role of different host factors involved in the movement of monopartite begomoviruses, specifically TLCV. In this section, the role of movement protein, coat

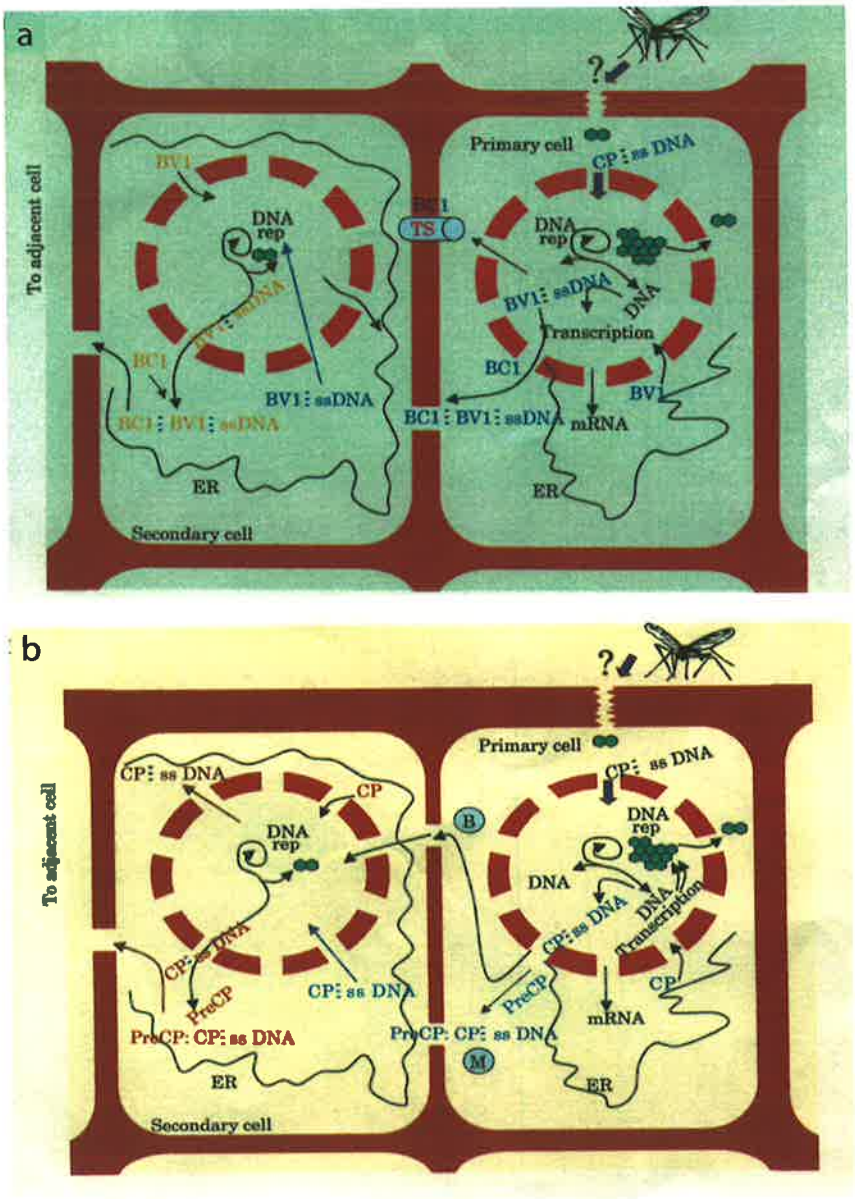


Figure 1.8. A model for geminivirus intra- and inter-cellular movement (Gafni et al., 2002)

(a) Virions of a bipartite geminivirus are introduced into the host cell by insect vector. A complex of the ssDNA and the CP is formed which enters the nucleus for replication and transcription of the viral genome. BV1 form a complex with ssDNA that the exits the nucleus and interacts with BC1. The BC1:BV1: ssDNA complex is directed to an adjacent cell. For SqLCV, it was suggested that BC1 associates with ER forming tubular structures (TS) which function in the translocation from cell-to-cell of the BV1: ssDNA complex. In subsequently infected cells, BV1 functions as a nuclear shuttle of the ssDNA.
(b) A complex of ssDNA and CP is formed and enters the nucleus for replication and transcription of the viral genome. CP is also proposed to function in nuclear export of the CP: ssDNA complex. Two routes are proposed to distinguish between the cell-to-cell movement of Mastrevirus (pathway M) which involves Pre-CP and CP, and that of begomoviruses (pathway B) for which only CP involvement has been conclusively demonstrated to date. Host protein involvement is not shown

protein and elements of the cytoskeleton and endomembrane system are discussed with particular emphasis on monopartite begomoviruses.

1.3.1 Movement Protein

To establish an infection, plant viruses encode proteins that help in cell-to-cell and long distance movement. Genetic studies have indicated that plant viruses can replicate and encapsidate in the absence of movement proteins (MP) but cannot move cell-to-cell. Many MPs have the ability to increase the size exclusion limit (SEL) and mediate cell-to-cell movement through the Pd (Lazarowitz & Beachy, 1999). Earlier studies indicated that MPs encoded by TMV and *Red clover necrotic mosaic virus* (RCNMV) could increase the size exclusion limit (SEL) of Pd (Citovsky et al., 1990; Fujiwara et al., 1993) and it appeared that cell-to-cell spread of infection could occur in the absence of viral particles when MP expressed in transgenic plants (Lazarowitz & Beachy, 1999). Therefore, it was suggested that complexes of viral nucleic acid and MP move cell-to-cell. Currently two distinct mechanisms have been described for inter-cellular movement through Pd (Lazarowitz & Beachy, 1999; Lucas & Gilbertson, 1994). The first mechanism suggests the interaction of MP with the viral nucleic acid to form an MP-nucleoprotein complex, which is then passed to the adjacent cell through Pd (McLean et al., 1997). The second mechanism describes the movement of virus in the form of virus particles through the specific tubular structures to the next uninfected cells (Kasteel et al., 1996). Specific and distinct interactions were observed between the cortical ER and the MPs encoded by TMV or SqLCV (Heinlein et al., 1998; Sanderfoot & Lazarowitz, 1995; Ward & Lazarowitz, 1999). It has been observed that MPs encoded by SqLCV and TYLCV begomoviruses accumulate in the nucleus (Rojas et al., 2001; Sanderfoot et al., 1996; Ward & Lazarowitz, 1999). Therefore, these findings

provide an additional dimension to MP function in nucleocytoplasmic transport and offer an opportunity to explore the regulation of nuclear shuttling and mechanism of nuclear export in plant cells. MP of SqLCV binds to the nucleic acids and assists the transport of these macromolecules through the plasmodesmata (Pascal et al., 1994). BV1 and BC1 in *Bean dwarf mosaic virus* (BDMV) interact directly with DNA and they have the unique property of recognising DNA on the basis of form and size rather than sequence (Rojas et al., 1998). *V1* ORF of TLCV encodes protein that is involved in cell-to-cell movement of TLCV (See section 1.9.1). TYLCV V1 and C2 mutants were not infectious in tomato (Wartig et al., 1997) and TYLCV V1 and C4 proteins exhibited cell-to-cell movement (Rojas et al., 2001). Collectively these results indicate that different viral proteins are involved in cell-to-cell movement and play a role as MPs. The role of different proteins encoded by TLCV involved in the viral movement is yet not clear.

1.3.2 Coat Protein

Viral genomes are encapcidated with viral protein known as coat protein (CP). In addition to its role as a protective protein, CP has been shown to be required for long distance movement in the majority of plant viruses. CP exhibits different roles in the case of geminiviruses. In contrast to monopartite geminiviruses, which require CP for infectivity, most of the bipartite geminiviruses do not require CP for systemic infection (Boulton et al., 1989; Liu et al., 1998; Rigden et al., 1993). Geminiviruses are transmitted by whiteflies, leafhoppers or a treehopper. The CP is required for insect transmission and determines vector specificity (Briddon et al., 1990). Virions of geminiviruses are injected into the host cells by insect vector. There is no other viral protein present in the cell except CP during initial infection. Therefore, CP seems to be

involved in the transport of ssDNA to the nucleus. Studies with MSV have shown that MP does not bind to viral DNA *in-vitro* but MSV MP interacts with MSV CP *in-vitro* (Liu et al., 2001) whereas, MSV CP interacts with the DNA and mediates nuclear transport of viral DNA (Liu et al., 1999). On the basis of these findings Liu et al. (2001) suggest that CP shuttles DNA to the nucleus, whereas CP-MP interaction involves the trafficking of viral DNA to the cell periphery for movement to the adjacent cell. The CP of TLCV is encoded by *V2* ORF (See section 1.1.6.3.2) (Dry et al., 1993). Experiments to determine the different functions of TLCV CP have not been performed.

1.3.3 Endomembrane system and cytoskeleton

The plant endomembrane system and cytoskeleton play a significant role in many intracellular transport processes during normal and pathogenic conditions. The endomembrane system constitutes ER, Golgi apparatus and associated vesicles (Carter et al., 2004). The plant cytoskeleton is mainly composed of a network of microtubules, microfilaments and diverse associated proteins (Aaziz et al., 2001). The cytoskeleton is involved in many biological processes like cell division and expansion, intracellular signals, organogenesis and tip growth. The cytoskeleton and ER play a pivotal role in viral trafficking (Reichel et al., 1999; Gafni & Bernard, 2002). Plant cells communicate with one another and disseminate nutrients through intercellular channels termed Pd (Zambryski & Crawford, 2000). It has been observed that MPs facilitate the passage of viral genomes through Pd. The most extensively studied viral MP is that of TMV. MP of TMV binds ssRNA (Citovsky et al., 1990), accumulates in (Roberts et al., 2001) and increases the size exclusion limits of Pd. Heinlein et al. (1998) have reported that TMV MP and replicases are localized to the ER and microtubules, thus these cytoskeleton elements are involved in viral pathogenesis. In contrast, it has been also suggested that

microtubules are not required for the cell-to-cell movement of TMV (Gillespie et al., 2002). Most of the information regarding role of cytoskeleton elements and endomembrane system in viral movement and localization are available in the case of RNA viruses. However, little information is available about geminiviruses particularly monopartite begomoviruses and there is a need to explore this area to discover the secrets of viral movement and localization.

1.4 Virus induced gene silencing

RNA silencing involves suppression of gene expression through a sequence-specific RNA in diverse eukaryotes. A unifying feature of RNA silencing is the cleavage of dsRNA into small interfering RNA (siRNAs; 21-26nt), which acts as an intermediate in the silencing pathway (Hamilton & Baulcombe, 1999). It has been shown that dsRNA is cleaved by a ribonuclease III-like enzyme called Dicer (Bernstein et al., 2001). In plants, four Dicer-like enzymes have been identified with distinct functions (Schauer et al., 2002). RNA silencing can be triggered by RNA viruses (Burton et al., 2000; Voinnet et al., 1999) and DNA viruses (Al-Kaff et al., 1998; Kjemtrup et al., 1998). Occurrence of RNA silencing is puzzling with DNA viruses which do not have a dsRNA in their replication cycle. Geminiviruses produce polycistronic transcripts by bidirectional transcription (Townsend et al., 1985). These polycistronic transcripts of opposite polarity could overlap at their 3' ends to generate dsRNA. Chellappan et al. (2004b) have confirmed this possibility by detecting the mRNA on either side using strand specific probing.

It has been observed that RNA silencing and virus resistance are related phenomena and viruses are both initiators and targets of a defence mechanism in transgenic and non-

transgenic plants (Ratcliff et al., 1997). Virus-induced gene silencing (VIGS) is a host defence mechanism triggered by dsRNA and targets both the viral (for geminiviruses, viral mRNA) and the endogenous RNAs. The idea that geminiviruses can also trigger this defence mechanism came from the use of geminivirus vectors (Kjemtrup et al., 1998; Peele et al., 2001). Recent reports suggest that DNA viruses, including geminiviruses, have the potential to initiate gene silencing (Chellappan et al., 2004b; Covey & Al-Kaff, 2000).

Viruses have evolved to fight back and to overcome the constraints imposed by gene silencing. To this end viruses have acquired the ability to produce proteins that function to suppress the host defence response. Helper Component-Protein (HC-Pro) encoded by *Tobacco etch virus* (TEV) and other potyviruses is able to suppress silencing in plants (Anandalakshmi et al., 1998; Brigneti et al., 1998). It has been demonstrated that the gene silencing suppressor, HC-Pro of the *Potato virus Y* (PVY), can also enhance accumulation of phloem limited PLRV in mesophyll tissues (Barker et al., 2001). The proteins encoded by *C2/AC2* ORFs of geminiviruses have a role as a gene silencing suppressor (Sunter et al., 2001; Voinnet et al., 1999). Similar results have been described with the C2 of the monopartite begomoviruses (Dong et al., 2003; Selth et al., 2004).

It seems that viruses are evolving strategies to evade the host defence mechanism. Transgenic resistance against geminiviruses has failed to produce high levels of success. There is a need to understand the virus-induced gene silencing (VIGS) mechanism with reference to viral localization at the cellular level.

1.5 Aims and significance of this project

Geminiviruses replicate through dsDNA (Fig. 1.9) in the nuclei of infected plant cells. Different forms of viral nucleic acids including ssDNA, dsDNA, sgDNA and transcripts are generated during the replication cycle of geminiviruses (Fig. 1.9). There is no conclusive report on the spatial and temporal localization of different forms of geminiviral DNA. Furthermore, geminiviruses trigger host defence responses which consequently generate viral specific siRNA during the replication process (Fig. 1.9) and regulate host gene expression. Replication of geminiviruses leads to expression of proteins required for viral replication, encapsidation and movement (Fig. 1.9). Analysis of movement related viral proteins and characterization of gene silencing at cellular level is also a focus area of this study.

1.5.1 Aims

The specific objectives described in this thesis are:

1 Establishing a double labelling in situ hybridization system to target viral and host factors at cellular level (Chapter3)

2 Tissue localization and intra-cellular identification of different forms of TLCV DNA in host plants (Chapter 4)

3 Analysis of viral proteins involved in the movement of TLCV (Chapter 5) by:

   (i) localization of defective virus having mutations in the ORFs involved in virus movement

   (ii) sub-cellular localization of TLCV encoded movement proteins

4 Investigation of silencing induced by TLCV at the cellular level (Chapter 6)

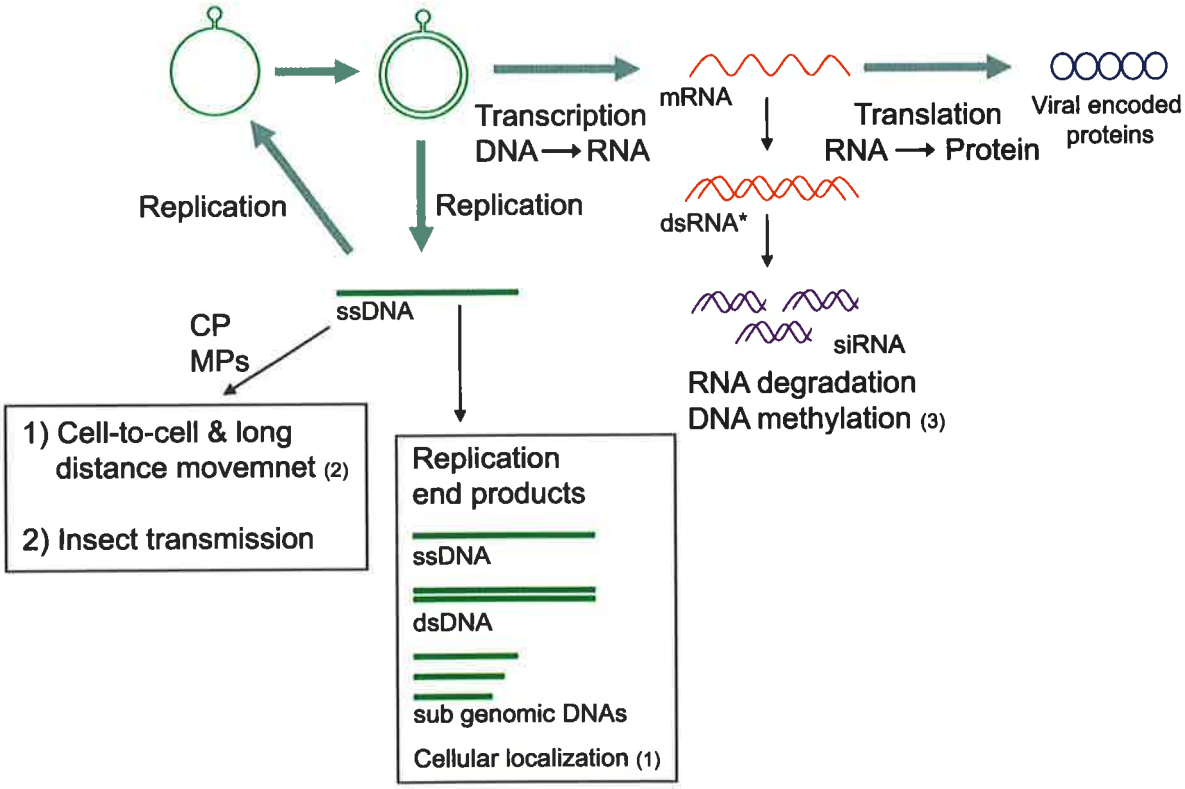


Figure 1.9. Life cycle of a geminivirus. ssDNA of a geminivirus replicates through ssDNA followed by gene expression to generate viral transcripts and to synthesise the viral encoded proteins required for viral replication, pathogenicity and movement. The host defence mechanism is activated by viral infection and produces viral specific siRNA. Virus ssDNA is produced from the dsDNA replicative form by a combination of rolling-circle replication (RCR) and recombination-dependent replication (RDR) mechanism. The newly synthesized ssDNA either reenters the replication cycle or spreads systemically in the form of nucleoprotein complex, or new virion particles, within the same host plant or to the next plant via insect transmission.

* as suggested by Chellappan et al. (2004)
(1) see chapter 4
(2) see chapter 5
(3) see chapter 6

5 Determining the localization of satellite DNA β molecule when it transreplicates with TLCV (Chapter 7)

1.5.2 Significance of the project

Geminiviruses are plant DNA viruses that infect a broad variety of economically important plants around the globe (Hanley-Bowdoin et al., 1999; Mansoor et al., 2003; Moffat, 1999). Although viral diseases associated with whiteflies have been recognized as a constraint to agricultural production since the early 1900s, the last two decades have seen a recurrence of begomoviruses as serious plant pathogens (Brown, 2000; Mansoor et al., 2003). Tomato-infecting geminiviruses cause severe crop losses in tomato growing regions of the world (see Section 1.1.6) (Nakhla & Maxwell, 1998). TLCV was first reported in 1970 in the Northern Territory, Australia (Behjatnia et al., 1996) and since then has caused severe to complete crop damage in the northern, tropical parts of Australia. TLCV is readily transmitted by the Australian indigenous biotype of *B. tabaci* (De Barro & Hart, 2000). Luckily this biotype occurs at low population densities and has a narrow host range (Stonor et al., 2003). However, in 1994 the silverleaf whitefly (SLW, *B tabaci* biotype B) was identified in Australia (Gunning et al., 1995). This insect, which is an efficient vector of TLCV, has spread to most mainland states and is well established in major horticultural regions of Queensland, causing significant crop damage through feeding on cotton, cucurbits, eggplants, navybeans, soyabenas, sunflowers, sweet potatoes and tomatoes (Stonor et al., 2003). Control of TLCV through management of SLW is impractical. Therefore, spread of TLCV to southern Queensland, a major tomato growing region, is predicted which could damage the tomato industry with an annual crop loss of $200-300 (Stonor et al., 2003). While this thesis was being written, an incursion of another monopartite

begomovirus, TYLCV, was reported in Queensland (Media release, Queensland Department of Primary Industry and Fisheries). This virus has spread rapidly to the major tomato regions and is likely to be a limitation to tomato production in Australia.

Effective control of TLCV by classical breeding strategies has not been successful (M. A. Rezaian, personal communication). Transgenic resistance based on pathogen-derived resistance has been reported against other geminiviruses, including TYLCV (Brunetti et al., 2001; Noris et al., 1996), TGMV (Day et al., 1991), ACMV (Hong et al., 1996), BCTV (Stenger, 1994) and SqLCV (Pascal et al., 1993). However, the results of engineered resistance against geminiviruses are inconsistent. Resistance against TLCV by genetic manipulation techniques using a range of constructs has not been achieved (M. A. Rezaian, personal communication). This study is expected to provide considerable insight into viral gene functions and to assist understanding the basic cellular plant response to TLCV. The information generated by this study will be utilized to devise new geminivirus control strategies aimed at impairing virus movement.

In addition to assisting devising strategies for disease control, this study on TLCV, which is a DNA virus, will contribute significantly to our understanding of basic molecular and cellular processes related to DNA replication, transcription and gene silencing.

Chapter 2 - General materials and methods

2.1 Materials

2.1.1 Solutions, chemicals and growth media

The solutions and growth media used in this project are outlined in Table 2.1. All chemicals were analytical or molecular biology grade and obtained from BDH or Sigma. The sources of all other supplies used in this project are indicated in the relevant methods section. Solutions were prepared with nanopure or deionised water and autoclaved where appropriate.

2.1.2 Oligodeoxyribonucleotides

Synthetic oligodeoxyribonucleotides were obtained from GeneWorks (Adelaide, South Australia). Their nucleotide sequences are shown in Table 2.2.

2.1.3 Bacterial strains

Escherichia coli strains XL1-Blue and DH5α (Stratagene; Cedar Creek, TX) were used for all routine cloning procedures. *E. coli* strain M15 (Qiagen; Clifton Hill, Australia) was used for recombinant protein expression. *Agrobacterium tumefaciens* strain C58 was used both to inoculate plants with gemininivirus infectious constructs and for infiltration experiments.

2.1.4 BY-2 cell cultures

Tobacco (*Nicotiana tabacum*) Bright Yellow-2 (BY-2) cells were used for sub-cellular localization studies of viral encoded proteins fused with GFP. The cells were generously

Table 2.1. Solutions and their compositions

Solution	Composition
DNA loading dye (10×)	78% glycerol, 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol, 10 mM ethylenediaminetetraacetic acid (EDTA)
RNA loading dye (10×)	50% (v/v) glycerol, 0.2M EDTA, 0.08% (w/v) bromophenol blue
Denaturing RNA loading buffer (5×)	70% (v/v) deionised formamide, 10% (v/v) formaldehyde, 6% (v/v) RNA loading dye (RNA), 14% (v/v) MOPS/EDTA buffer
Formamide loading buffer	Deionized formamide, 0.1% (w/v) bromophenol ble, 0.1% xylene cyanol
MOPS/EDTA buffer (10×)	200 mM MOPS, 50 mM sodium acetate, 10 mM EDTA, pH 7.0
Murashige and Skoog (MS) salt solution	4.33 g MS basal salt mixture (comp.)/L
BY-2 culture media	1x MS_0 (Murashige and Skoog), 1x vitamins B5 and 2 mg l^{-1} 2,4-D, pH 5.8
DNA extraction buffer	50 mM Tris (pH 8.0), 100 mM NaCl, 10 mM EDTA (pH 8.0), 1% (w/v) SDS, 1.5% (v/v) β-mercaptoethanol.
RNA extraction buffer	50 mM Tris (pH 8.0), 100 mM NaCl, 5 mM EDTA (pH 8.0), 2% (w/v) SDS,
LB agar (solid growth media)	1% (w/v) bacto-tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl, 1.2% (w/v) Bacto-agar, pH 7.0
PBS	138 mM NaCl, 10mM sodium phosphate (pH 7.4), 2.7mM KCl
PBS-Tween	138 mM NaCl, 10mM sodium phosphate (pH 7.4), 2.7mM KCl, 0.05% Tween-20
TBST (10×)	1.5 M NaCl, 0.2 M Tris HCl (pH 7.5), 50 ml 20% Tween 20
GUS assay buffer	50 mM phosphate buffer (pH 7.0), 0.1% Triton X-100, 1 mM X-gluc (5-bromo-4-chloro-3-indolyl-b-D-glucuronic acid)
GUS fixing buffer	5% (v/v) formaldehyde, 5% (v/v) acetic acid, 20% (v/v) ethanol
Hybridisation buffer	0.25 M sodium phosphate buffer (pH 7.2), 7% SDS (w/v), 1 mM EDTA
GUS extraction buffer	50 mM phosphate buffer (pH 7.0), 10 mM β-mercaptoethanol, 10 mM EDTA (pH 8.0), 0.1% sodium lauryl sarcosine, 0.1% Triton X-100
MUG assay buffer	40% methanol, 60% GUS extraction buffer, 1 mM methylumbelliferyl-beta-D-glucuronic acid (MU)
Stop buffer (MUG assay)	0.2 M Na_2CO_3
SSC	150 mM NaCl, 15 mM tri-sodium citrate, pH 7.0
STET buffer	8% (w/v) sucrose, 5% (v/v) Triton X-100, 50 mM EDTA (pH 8.0), 50 mM Tris (pH 8.0)
TBE buffer	90 mM Tris, 90 mM borate (pH 8.3), 2 mM EDTA
TE buffer	10 mM Tris, 1 mM EDTA, pH 8.0
SDS-PAGE sample loading buffer	0.5 M Tris (pH 6.8), 10% glycerol, 2% SDS, 5% β-mercaptoethanol, and 0.05% bromphenol blue
STE buffer (10×)	500 mM Tris (pH 6.85), 1 M NaCl, 10 mM EDTA
Ni-NTA binding buffer	50 mM NaH_2PO_4 (pH 8.0), 300 mM NaCl, 10 mM β-mercaptoethanol, 1% Tween-20
Ni-NTA elution buffer	50 mM NaH_2PO_4 (pH 8.0), 300 mM NaCl, 10 mM β-mercaptoethanol, 1% Tween-20, 250mM imidazole, 10% glycerol
Ni-NTA washing buffer	50 mM NaH_2PO_4 (pH 8.0), 300 mM NaCl, 10 mM β-mercaptoethanol, 1% Tween-20, 20mM imidazole, 10% glycerol
Electro-transfer buffer	200mM glycine, 25mM Tris, 20% methanol

Table 2.2. Oligonucleotide primers used in this study

Primer	Description	Sequence (5'→ 3')
P1	TLCV V1-F	GG**GAATTC**TGGGATCCTTTAGTCCAC[a]
P2	TLCV V1-R	GGTT**CTCGAG**TCAGGGCTTCTGAACAGC[a]
P3	TLCV V2-F	GG**GAATTC**AGCAAGCGACCAGCAGAT[a]
P4	TLCV V2-R	GG**GGATCC**TTAATTCTGAATCGA[a]
P5	TLCV C4-F	TT**GAATTC**ATGAGAATGGGGAGCCTC[a]
P6	TLCV C4-R	GT**GGATC**CATTCCCTAAGGACGTTA[a]
P7	TLCV IR-F	TCG**GAGCTC**GTGTCTGGGGTCTTAT[a]
P8	TLCV IR-R	GGGCCCAAGTATATATACGACAAAAAAC
P9	TGMV AV1-F	GG**CATATG**CAAGATATGGATGGATG[a]
P10	TGMV AV1-R	TCCTAACCAGAGCCTGCTCGTTG
P11	TGMV AC1-F	CCG**CATATG**GCCGCGCAGCGGA[a]
P12	TGMV AC1-R	CTAACGACGCTGCAGCAGAGGCGT
P13	TYLCSV V1-F	CCA**CTGCAG**ATGCCGAAGCGAACC
P14	TYLCSV V1-R	CCGTTAATTTGTTACAGCATCATAAAAAT AA
P15	TYLCSV C4-F	CCG**CTGCAG**ATGAAAATGGGGAACC[a]
P16	TYLCSV C4-R	CCGTTACATCAAGAGCCTGCGACTTA
P17	Ubiquitin-F	GGGATGCAGATCTTCGTGAAAACCC
P18	Ubiquitin-R	TCAATCGCCTCCAGCCTTGTTGTAA
P19	GFP-XhoI-F	GCG**CTCGAGA**TGAGTAAAGGAGAAG
P20	GFP-SpeI-R	CGC**ACTAGT**TTATTTGTATAGTTCATCC
P21	V1-SpeI-R	CCG**ACTAGT**TCAGGGCTTCTGAACA
P22	V2-SpeI-R	CCCACTAGTTTAATTCTGAATCGAATCA
P23	C4-XhoI-F	AATCTCGAGATGAGAATGGGGAGCC
P24	C4-Meth-F	tAgGTtTTtAAAGtAAGTGGATTGATGTGAt ATttAt[b]
P25	C4-MethI-R	CCCTTATCccAAAACTACTCACACATTATT TATAAaa[b]
P26	C4-MethII-R	CACATTATTTATaaAaAAAATAaAaAaAaAT AaATTTaTA[b]
P27	IR-Meth-F	ACATAATCCTTAaaaaCTAATTCCCTAAaaA CcTTAAaAa[b]
P28	C1-Meth-R	TTAttAAAATGttATAgGGTGTttAAtTATAAA TAAGatt[b]
P29	βC1-F	CCG**CATATG**TCATACATCTGAATTCAT
P30	βC1-R	CCGATGACAATCAAATACAACAACATG
P31	CLCuV AC1-F	TTT**CATATG**GGGCCCCCATGAACTC
P32	CLCuV AC1-R	AAATCTAGACAGAATCTTTCAGGAGCC
P33	SlUPTG1-EcoRI-F	TT**GAATTC**ATGGCAGCAGCAACACCA
P34	SlUPTG1-BamHI-R	GT**GGATCC**CTTTTTAGTCTTTGCTGG

[a]Bold sequences correspond to specific restriction enzyme sites.
[b]Lower case g and c denote bases complementary to methylated C in the DNA strand being sequenced or in its complementary-sense strand, respectively. Lower case t and a denote bases complementary to unmethylated C in the DNA strand being sequenced or in its complementary strand, respectively.

provided by Dr Roger N Beachy (Donald Danforth Laboratories, St Louis, MO 63132, USA). The BY-2 cultures were grown in 50 ml of BY-2 culture media (Table 2.1) in 250 ml culture flasks on an orbital shaker set at 100-110 rpm in the dark at 26 ± 2°C. The cultures were maintained by subculturing weekly with a 1:10 dilution of the original culture.

2.2 Methods

This section outlines general methods used throughout this project. Most procedures are essentially as described by Sambrook and Russell (2001) or according to the manufacturer's instructions. Methods which have been significantly modified from their published form are outlined. Other specific protocols relevant to particular experiments are outlined in the corresponding chapters.

2.2.1 Restriction endonucleases and digestion of DNA

DNA was digested with restriction endonucleases obtained from Roche Diagnostics (Indianapolis, IN), Promega (Annandale, Australia), New England Biolabs (Beverly, MA), and Fermentas (Hanover, MD) using buffers supplied by the manufacturers.

2.2.2 Gel electrophoresis

2.2.2.1 Standard agarose gel electrophoresis for DNA and RNA

Agarose gels were prepared from 0.7-2.0% (w/v) using Type I-A low EEO agarose in TBE buffer (Table 2.1), and contained 0.5 μg/ml (w/v) ethidium bromide. Horizontal minigel tanks (EasyCast Electrophoresis system, OWL Scientific Inc., Cambridge, UK) were used for electrophoresis of DNA. Samples were adjusted to 2× DNA loading dye (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× TBE running buffer (Table 2.1) at approximately 100 V before being visualised and photographed using a short wavelength UV transilluminator.

Electrophoresis of RNA was essentially the same as that described for DNA except that gel tanks, trays, and combs were treated with 0.4 M NaOH for approximately 1-2 h prior to use to inactivate RNases. RNA loading dye (Table 2.1) was added and each sample was heated at 65°C for 5 min and then immediately chilled on ice for 2 min before loading into a well.

2.2.2.2 Denaturing agarose gel electrophoresis for RNA gel blot analysis

Denaturing agarose gels were prepared by adding the appropriate amount of agarose to 105 ml of water, autoclaving, adding 30 ml 37% formaldehyde and 15 ml sterile 10× MOPS/EDTA buffer (Table 2.1), and then pouring into a gel tray pre-treated with 0.2 M NaOH. Samples were adjusted to 3× denaturing RNA gel loading buffer (Table 2.1) and incubated at 65°C for 15 min before loading. Electrophoresis was carried out in 1× MOPS/EDTA buffer (Table 2.1).

2.2.2.3 Polyacrylamide gel electrophoresis

Denaturing polyacrylamide gels were prepared for RNA analysis by adding 15% (w/v) acrylamide (19:1 acrylamide:bis; BioRad, USA) , 1 x TBE and 7 M urea. 0.6 mg ml^{-1} freshly prepared ammonium persulfate and 0.5 µl of TEMED per 1 ml of the solution was added to initiate the polymerization. The gel was cast using plates (90 x 70 x 1 mm) suitable for use in the Mini-PROTEAN II electrophoresis module (Bio-Rad, USA). The gel wells were rinsed several times with 1× TBE to minimize urea accumulation in the

wells. RNA samples were adjusted to a minimum of 0.4x formamide loading buffer (Table 2.1) and denatured by heating at 95°C for 3-4 min before loading on the gel. The samples were electrophoresed at 25-30 mA in 1× TBE until the bromophenol blue dye band had reached the bottom of the gel casting plates. To visualize the RNA bands, the gels were soaked in a solution of 1× TBE containing 2 μg ml^{-1} ethidium bromide for 15 min with gentle agitation and photographed using a short wavelength UV transilluminator.

2.2.3 Purification of DNA from agarose gel slices

Extraction of specific DNA bands from agarose gels after visualization with ethidium bromide was done by using a QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer's instructions.

2.2.4 DNA amplication by the polymerase chain reaction (PCR)

DNA species to be cloned were amplified by PCR with Platinum Taq DNA Polymerase High Fidelity (Invitrogen; Carlsbad, CA) using 1× High Fidelity reaction buffer and 1.5 μM $MgSO_4$ (Invitrogen). For all other purposes, components of a PCR reaction were as follows: DNA template (~ 10ng), oligonucleotide primers (200-500 nM), 1× reaction buffer (Gibco BRL; Rockville, MD), 200 μM dATP, dCTP, dGTP, and TTP (Promega), 1.5 μM $MgCl_2$, and 0.5 units of recombinant Taq DNA polymerase (Gibco), in a 20 μl reaction volume. Thermal cycling generally consisted of: 3 min at 95°C (one cycle); 45 s at 94°C, 35s at 55°C, 50 min at 72°C (35 cycles); 10 min at 72°C (one cycle).

2.2.5 Dephosphorylation of DNA 5' termini

The 5' phosphate groups were removed from restricted vector DNA prior to ligation with inserted DNA fragments using calf intestinal alkaline phosphatase (Roche). The enzyme was inactivated by adding 20 mM EDTA and heating at 65°C for 15 min.

2.2.6 Converting 5' or 3' DNA overhangs to blunt ends

To convert 5' or 3' overhangs to blunt ends, up to 5 μg of the DNA of interest was mixed with 100 μM dNTPs, 1× restriction enzyme buffer B (Promega) and 5 units of T4 DNA polymerase (Promega) per μg of DNA. The reaction mix was incubated for 30 min at room temperature.

2.2.7 Extraction of DNA samples following enzymatic reactions

Extraction of DNA samples after restriction enzyme digestion (Section 2.2.1), PCR (Section 2.2.4), dephosphorylation (Section 2.2.5) and blunting (Section 2.2.6) reactions was achieved using a QIAquick PCR Purification Kit (Qiagen). Where applicable, nucleic acids in enzymatic reactions were recovered by adjusting the reaction volume to 500 μl with deionized water and adding an equal volume of phenol:chloroform:isoamylalchohol (25:24:1). The mixture was vortexed for 2 min and centrifuged at 16000 *g* for 10 min. The supernatant was added to an equal volume of chloroform:isoamylalchhol (24:1), vortexed and centrifuged as above. The supernatant was then ethanol precipitated (see section 2.2.8) and the nucleic acid pellet resuspended in either TE buffer or deionized water.

2.2.8 Ethanol precipitation of nucleic acids

Solutions containing DNA and/or RNA were precipitated by adding 1/10th 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 over 30 min. The precipitate was recovered by centrifugation at 16000 *g* for 20 min. The pellet was washed with 70 % ethanol, dried in a vacuum and resuspended in either TE buffer or deionized water.

2.2.9 DNA ligation

Purified PCR products were ligated into the T-tailed vectors pGEM T-Easy (Promega) or pDRIVE (Qiagen) according to the manufacturer's instruction. All other ligations, including blunt-end ligations, were carried out in 10 µl reaction volumes containing a insert:vector molar ratio of approximately 3:1 and 3 units of T4 DNA ligase (Promega) in the supplied buffer and incubated overnight at 16°C.

2.2.10 Transformation of bacteria with recombinant plasmids

Electrocompetent *E. coli* XL1-Blue, *E. coli* DH5α, *E. coli* M15, and *A. tumefaciens* C58 cells (Section 2.2.10) were transformed by electroporation using a Gene-Pulser apparatus (Bio-Rad, Hercules, CA). Approximately 5 ng of plasmid or 1 µl of ligation reaction mixture was added to a 25-50 µl aliquot of electrocompetent cells and transferred to an ice-cold electroporation cuvette (path length = 1mm; Invitrogen). The cuvette was placed into the Gene-Pulser electroporator set at 1.8 kV, 125 µFD and 200 Ohms and immediately given a single pulse. The cells were resuspended in 400 µl of LB (Table 2.1). After incubation at 37°C for 1 h to allow expression of antibiotic-resistance genes, the transformed cells were spread on 1.2% LB agar plates (Table 2.1) with appropriate antibiotic selection and incubated at 37°C overnight.

2.2.11 Preparation of electrocompetent *E. coli* cells

LB (500 ml) was inoculated with a five ml overnight culture of the *E. coli* strain of interest and grown at 37°C with vigorous shaking to an optical density (OD_{600}) of 0.5. Cells were chilled on ice for 10 min and centrifuged for 15 min at 4°C at 5000 *g*. The cells were resuspended in 500 ml of sterile ice-cold water and centrifuged again. The cells were washed and centrifuged again with 250 ml sterile ice-cold water and resuspended in 10ml of sterile ice-cold 10% (v/v) glycerol. Bacterial cells were transferred to a new 50 ml falcon tube and centrifuged again. The cells were finally resuspended in 2 ml of ice-cold 10% glycerol. Aliquots of 25 µl were placed into ice-cold microcentrifuge tubes, snap-frozen in liquid nitrogen, and stored at -80°C.

2.2.12 Growth of bacteria in liquid cultures

Liquid cultures were set up by inoculating LB containing appropriate antibiotics with a single bacterial colony or one loopful of frozen glycerol stock. Cultures were incubated at 37°C (*E. coli*) or 28°C (*A. tumefaciens*) overnight with shaking.

2.2.13 Preparation of bacterial glycerol stocks

Where required, glycerol stocks of bacterial cultures were prepared by adding 1 volume of 40% or 80% sterile glycerol to an overnight culture, snap-freezing in liquid nitrogen, and storing at –80°C.

2.2.14 Preparation of bacterial plasmid DNA

High quality plasmid DNA for vector construction, transformation of competent bacterial cells, DNA sequencing, transient expression in onion epidermal cells and BY-2 cells was prepared from 1-5 ml of overnight culture using a QIAprep Spin Miniprep

Kit (Qiagen) according to the manufacturer's directions. Large-scale (20-50 ml cultures) preparation of plasmid DNA was achieved using a Plasmid Midi Kit (Qiagen).

For general screening purposes, plasmid DNA was prepared using a miniprep boiling method (Holmes & Quigley, 1981). Briefly, 1 ml of overnight culture was centrifuged at 16,100*g* for 1 min at room temperature. The supernatant was discarded and the pellet resuspended in 350 µl STET (Table 2.1) buffer. Then 12.5 µl of 20 mg/ml lysozyme was added to the cells and mixed gently. Immediately after adding the lysozyme, cells were heated to 100°C for 1 min and centrifuged at 16,100 *g* for 20 min. The pellet was removed with a sterile toothpick and 40 µl 3 M sodium acetate and 220 µl isopropanol added to the supernatant. This mixture was centrifuged at 16,100 *g* for 10 min. The resulting pellet was washed with 70% ethanol, dried under vacuum, and resuspended in 50 µl of sterile water.

2.2.15 Immunoblotting of proteins expressed in bacteria

Immunoblotting was used to detect transiently expressed viral:GFP fusion protein in agroinfiltration experiments. Protein samples were first electrophoresed in 4-20% Tris-glycine-SDS polyacrylamide gels (Life-Gels, Clarkston, GA) at 150 V for 1-1.5 h. The gel was assembled into a sandwich as follows: gel-sized sponge, two pieces of gel-sized Whatman 3MM paper, gel, 0.45 µm Immobilon P polyvinylidene fluoride (PVDF) membrane, two pieces of gel-sized Whatman 3MM paper, and another gel-sized sponge. The sandwich was placed into a transfer tank containing 1 L of transfer buffer (Table 2.1) with the gel side facing the negative electrode and transferred at 150-250 mA for 1.5-3 h. After disassembling the sandwich, the membrane was placed into a clean container and washed with distilled water for 5 min with gentle agitation. Blocking was

carried out in 20 ml PBS (Table 2.1) containing 5% skim milk powder for at least 2 h with gentle agitation. After washing the membrane for 3× 5 min with PBS-Tween, anti-V1 or anti SlUPTG1 antibody were applied at a concentration of 1:4,000 in PBS-Tween containing 3% milk powder and incubated with the membrane for 1-2 h with gentle agitation. After washing the membrane for 3× 5 min with PBS-Tween, goat anti-mouse IgG-alkaline phosphatase (AP) conjugate was applied at a concentration of 1:20,000 in PBS-Tween containing 3% milk powder and incubated with the membrane for 1-2 h with gentle agitation. The membrane was washed again for 3x 5 min with PBS-Tween and AP activity detected using SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology, Rockford, IL).

2.2.16 Mini DNA extraction

Emerging leaves were selected and 150 mg tissue was placed in eppendorf tubes and frozen in liquid nitrogen. The tissue was ground briefly in eppendorf tube by electrical drill using a plastic pestle. 400 µl of DNA extraction buffer (Table 2.1) and a small amount of sterile sand was added and the mixture was ground thoroughly for 1 min. After adding 400 µl phenol:chloroform (4:1) and briefly vortexing, tubes were centrifuged at 16,100 *g* for 10 min. Supernatant was extracted two more times with 400 µl of phenol:chloroform (4:1) and finally with an equal volume of chloroform:IAA (24:1). The resulting supernatant was ethanol precipitated (see section 2.2.8). The pellet was washed with 500 µl 70% ethanol, dried, and resuspended either in 100 µl TE or deionized water. 1 µl was used for PCR or 10 µl was used for southern hybridization.

2.2.17 Total RNA extraction

Total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen) if the sample size was less than 100 mg. For larger sample sizes, 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 phenol equilibrated with 0.2M Tris-HCl (pH 8.0). The mixture was agitated vigorously for 10 min at room temperature and centrifuged at 3,920 *g* in a bench top centrifuge for 10 min. The supernatant was extracted twice with phenol and once with chloroform:IAA (24:1) and the RNA recovered by ethanol precipitation (see section 2.2.8). The RNA pellet was resuspended in 100-500 µl TE or deionized water.

2.2.18 Preparation of DNA samples for sequencing

DNA sequencing reactions were carried out using an ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Norwalk, CT). Sequencing reactions consisted of 8 µl of Terminator Ready Reaction Mix, 3.2 pmol oligonucleotide primer, DNA template (300-500 ng dsDNA or 25-100 ng PCR-generated DNA) and deionized water to 20 µl. The reactions were briefly vortexed and 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 and reactions were held at 4°C until purification. The reaction products were precipated by adding 80 µl of 75% isopropanol, incubating for 20 min at room temperature, and centrifuging at 16,100 *g* for 20 min. After discarding the supernatant and adding another 250 µl of 75% isopropanol, the tubes were centrifuged at 16,100 *g* for 5 min. The samples were then aspirated, dried under vacuum for 15 min, and sent to the Institute of Medical and Veterinary Science (Adelaide, Australia) for analysis.

2.2.19 Sequence analysis and manipulation

DNA sequences were analysed using basic local alignment search tools (BLAST) at the National Centre for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/blast/) and the GCG WebAngis Wisconsin software package operated by Australian National Genomic Information Service (http://www.angis.org.au/).

2.2.20 Synthesis of ^{32}P-labelled nucleic acid probes

Probes for hybridisation analysis were synthesised by random priming using the Rediprime II DNA Labeling System (Amersham Biosciences, Little Chalfont, England). 2.5-25 ng of template DNA diluted in 45 µl TE buffer was heated at 95°C for 5 min, chilled on ice for 5 min, and labelled with 5 µl of Redivue stabilised [α-^{32}P]dCTP (3000 Ci/mmol; Amersham Biosciences) at 37°C for 10-30 minutes. Prior to hybridisation, unincorporated dNTPs were removed by passing the probe though a ProbeQuant G-50 Micro Column (Amersham Biosciences) according to the manufacturer's instructions and denatured by heating at 95°C for 5 min.

2.2.21 RNA gel blot analysis

Total nucleic acid was extracted from plant tissue as described by Selth *et al.* (2004). Nucleic acid samples of 2-20 µg were denatured with denaturing buffer (Lewandowski & Dawson, 1998) for 10 min at 65°C, cooled on ice, mixed with loading buffer and analysed on 1.2% agarose MOPS gels containing 2 M formaldehyde (Section 2.2.2.2). RNA from the gel to Zeta-Probe membrane (Bio-Rad) was transferred overnight in 10× SSC using a TurboBlotter (Schleicher & Schuell, Dassel, Germany). The RNA was stabilised on the membrane by cross-linking using a UV Stratalinker™ 1800

(Stratagene, La Jolla, CA). RNA on membranes was hybridised with specific ^{32}P labelled probes (Section 2.2.22) in 5-15 ml of hybridisation buffer (Table 2.1) at 65°C overnight. Membranes were washed twice at 65°C in 2× SSC/0.1% SDS and once at 65°C in 0.1× SSC/0.1% SDS. Radioactively labelled bands were detected by autoradiography using Biomax MS Scientific Imaging Film (Kodak, Rochester, NY).

2.2.22 Dot blot hybridisation

Dot-blot hybridisation was used as a quick, semi-quantitative method to assess the relative levels of TLCV genomic DNA in plants. 100 mg of tissue obtained from emerging leaves was ground in 200 µl of 0.5 M NaOH with a small amount of sterile sand, left for 30 min at room temperature, and then centrifuged at 16,100 *g* for 10 min. 4 µl of the supernatant was dotted onto Zeta-Probe membrane, which was allowed to air dry, washed once with chloroform and twice with 2× SSC, and cross-linked. TLCV sequences were detected by hybridisation and autoradiography as described in Sections 2.2.21.

2.2.23 Southern blot hybridization analysis

DNA samples were fractionated in agarose gels (1-1.5%, see section 2.2.2.1) and blotted onto a Zeta-Probe nylon membrane (Bio-Rad) by a rapid downward transfer system (Schleicher and Schuell, USA) according to the manufacturer's instructions. The membrane was washed in 2× SSC for 5 min with gentle agitation, and cross-linked. The membrane was air-dried and stored in a plastic resealable bag, or used immediately. Hybridization and autoradiography were done as described in Sections 2.2.21.

2.2.24 Analysis of GFP-fusion proteins by microprojectile bombardment

The pTA7002 plasmid with a glucocorticoid inducible promoter (Aoyama & Chua, 1997) and pART7 (Gleave, 1992) were used as vector to transiently express GFP fusion proteins in onion tissue after microprojectile delivery. Onion epidermal strips on agar containing Murashige and Skoog (MS) Salt Mixture (Invitrogen) were bombarded with vectors. For four shots, 400 µg of gold particles in 100 µl ethanol were vortexed for 2 min, spun down for 10 s in a microcentrifuge, drained, washed twice with sterile water, and resuspended in 25 µl 40% glycerol. While gently vortexing, 4 µl of the plasmid solutions (400 ng/µl), 10 µl of cold 0.1 M spermidine and 25 µl 2.5 M $CaCl_2$ were added dropwise and the resulting mixture incubated on ice for 10 min. The particles were spun down, washed with 70% ethanol, resuspended in 24 µl cold 100% ethanol, and 6 µl aliquots were placed onto sterile filter holders. After sterilising the gun chamber with 70% EtOH, plates containing onion strips on MS media were placed inside, covered with a sterile mesh, and bombarded with a pressure of 650 kPa after evacuating the chamber to 90 kPa. After bombardment, tissue was stored in the dark for 24 h.

2.2.25 Immunofluorescence by Laser Scanning Confocal Microscopy (LSCM)

The images were produced using the BioRad Radiance 2100 confocal microscope (The Hanson Detmold Imaging Core Facility, Institute for Medical and Veterinary Science, Adelaide, Australia) equipped with three lasers, Argon ion 488nm; Green HeNe 543 nm; Red Diode 637 nm and Olympus IX70 inverted microscope. The objective used was a 20× UPLAPO (NA= 0.70) or 40× UPLAPO with NA=1.15 water, or a 60× UPLAPO with NA=1.4 water. The single or dual labelled samples were imaged with two separate channels (PMT tubes) in a sequential setting. The Green fluorescence

(GFP) was excited with Ar 488 nm laser line and the emission was viewed through a HQ515/30 nm narrow band barrier filter in PMT1. The red fluorescence (Alexa 546) was excited with Green HeNe 543 nm laser line and the emission was viewed through a long pass barrier filter (570LP) to allow only red light wavelengths longer than 570 nm to pass through PMT2. Automatically all signals from PMTs 1 and 2 were merged. The image data were stored on a CD for further analysis using a Confocal Assistant software program for the Microsoft® Windows™ (Todd Clark Brelje. USA).

Chapter 3 – Double labelling in situ hybridization using plastic embedded plant tissues

3.1 Introduction

In situ hybridization (ISH) is a robust technique for the detection of specific nucleic acid sequences and analysis of gene expression during development processes (McFadden, 1989; Wilkinson, 1992). Most importantly ISH provides information both on cellular and molecular levels in a morphological context. The technique was originally described by Gall and Pardue (1969) and John et al. (1969). Initially, radioactively labelled probes were utilized for the detection of target nucleic acids (McFadden, 1989; McFadden et al., 1988; Wilkinson & Green, 1990). Over recent years, development of non-isotopic labelling systems and detection methods has revolutionised the technique (Abraham, 2001; Butler et al., 2001). The principal of ISH is the same as for other nucleic acid hybridization methods with some modifications. The major difference is that ISH involves the hybridization of nucleic aids located within fixed cells. Therefore, certain parameters must be applied to ensure a successful result. The following of specific conditions are essential for high quality results. First, the integrity of target nucleic acid within the cell must be retained. Second, the probe must have access to the target nucleic acid for hybridization. Third, the hybridization reaction must be detectable. Fourth, the signal-to-noise (non-specific hybridization) ratio should be minimal. Finally, the structure of tissues and cells must be maintained. Recently developed fixation conditions, embedding procedures and quality of probes have significantly enhanced the sensitivity of ISH.

Here a modified protocol of in situ hybridization is described which allows determination of the spatial and temporal expression patterns of viral nucleic acids, transgenes and host genes. The modified ISH provides an opportunity to understand the biology and life cycle of geminiviruses at the cellular level and the identification of host factors upon viral infection. The modifications described here include strand specific probing, double labelling, enzymatic digestion of transcripts and hybridization under denaturing and non-denaturing conditions. Furthermore, tissues embedded in plastic resin were used to give better resolution than wax embedded tissues. Some of these techniques are novel for research in plant virology.

3.2 Materials and Methods

Solutions used in the ISH procedure were prepared in sterilized deionized water and, where applicable, solutions were filter sterilized. All other material including tubes, glassware, pipette, and stirrers were RNase free. Gloves were worn throughout the procedure, cleaned with RNase ERASE (MP Biomedicals, Aurora, Ohio) and changed frequently. All steps from fixation until the posthybridization washes were performed under RNase free conditions. The flow chart of the ISH procedure with a time period for each step, and the principal of ISH are outlined in Figure 3.1.

3.2.1 Viral infectivity assays

Nicotiana benthamiana plants were grown under glasshouse conditions at 25-30°C. Plants at the 4-5 leaf stage were inoculated with an overnight culture of *Agrobacterium tumefaciens* (strain C58) containing infectious viral clones (Rasheed et al., 2006; Grimsley et al., 1987). Developing leaves from individual plants (50 ~ 100 mg) were sampled and homogenized with 0.5 M NaOH at a ratio of 4 µl buffer per 1 mg tissue.

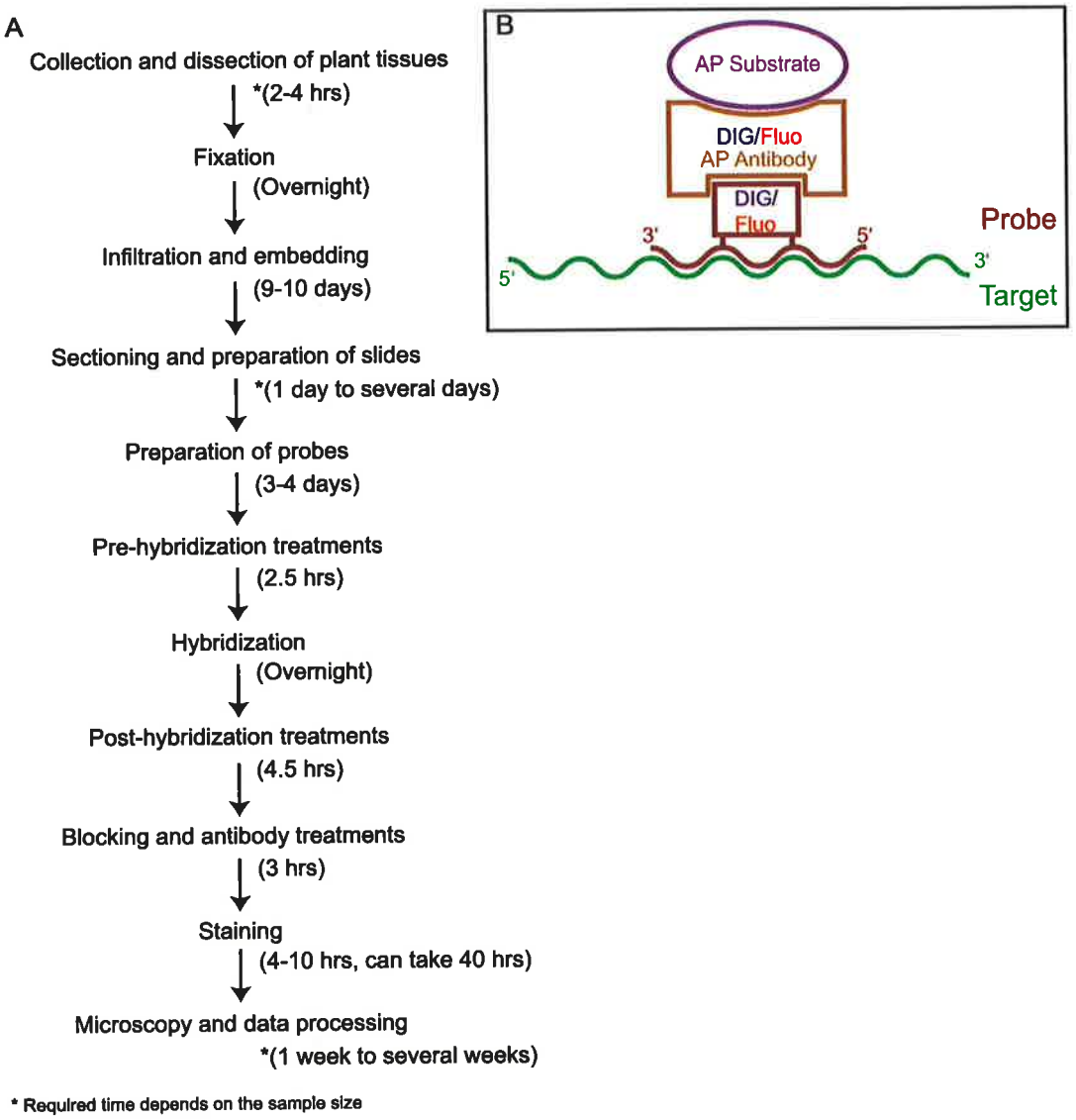


Figure 3.1. In situ hybridization: methodology and principle. **(A)** A flow diagram of the in situ hybridization protocol describing the stepwise procedure with approximate time period for each treatment. **(B)** Schematic representation of in situ hybridization based on the enzymatic detection procedure.

The presence of viral DNA was tested by dot-blot analysis (Stonor et al., 2003). Young symptomatic leaves were collected 3-4 weeks post-inoculation and processed for fixation as described below.

3.2.2 Plant material and fixation

Plant material of interest was collected and tissues were dissected into 4 mm by 6 mm pieces in cold 1× PBS (Table 3.1). It was important not to take larger pieces as they would not fix properly and would crumble during sectioning. Tissue pieces were placed on ice during sectioning and then were transferred to a vial containing fixative solution (Table 3.1). They were completely submerged in the fixative solution (Fig. 3.2) for 2 hours at 4°C. The solution was replaced with a fresh batch of fixative solution and vials with loosened caps were placed at low pressure (70 kPa) for 15-20 minutes. The low pressure infiltration treatment was repeated 3-4 times and vials were put at 4°C overnight. Next day tissues were washed twice for 10 minutes each on ice with 0.05M Na phosphate buffer pH 7.2 containing 1mM DTT and subsequently were dehydrated on ice in a graded aqueous 10% to 100% ethanol series containing 1mM DTT for 30 minutes each treatment. Tissues at this stage could be stored in 70% ethanol at -20°C for a longer period (1-24 months) without detrimental effects. Tissues were subjected to two additional dehydration treatments in 100% ethanol containing 1mM DTT for 1 hour each to ensure complete dehydration before infiltration.

3.2.3 Infiltration and embedding

Tissue was infiltrated at 4°C in a graded series of ethanol (EtOH) in BMM (Table 3.1). The infiltration process was as follows: 2-4 hours (or overnight) in EtOH/DTT: BMM (3:1 v/v), 2-4 hours (or overnight) in EtOH/DTT: BMM (1:1 v/v), 2-4 hours (or

Table 3.1. Solutions and their compositions

Solution	Composition
PBS (10×)	1.3 M NaCl, 0.07 M Na_2HPO_4, 0.03 M NaH_2PO_4
Fixative solution	4% paraformaldehyde in 1x PBS containing 0.25% glutaraldehyde and 1mM DTT; to dissolve paraformaldehyde in PBS, the pH of PBS was brought to 11 with 10 N NaOH and heated to 60°C. 8gm of paraformaldehyde was added to PBS in the fume hood and the bottle was shaken vigorously for about 30 seconds and pressure was released every 5-10 seconds. Once paraformaldehyde was dissolved in PBS, the solution was cooled on ice and 200µl of 1MDTT was added. The pH of the solution was brought to pH 7 using conc. H_2SO_4. HCl should not be used because it can release a carcinogen. 0.25% (500 µl/200 ml) glutaraldehyde was added to the solution and filtered through sterilised Whatman 1 paper into a sterile bottle. The fixative solution was kept on ice until use.
BMM	40 ml of n-butyl methachrylate (ProSciTech), 10 ml of methyl methachrylate (ProSciTech), 250 mg (0.5% w/v) benzoin methyl ether (ProSciTech), 0.008 g DTT (1mM); the solution was bubbled very gently with N_2 or argon for 15 minutes two times at 10 minute intervals to displace dissolved oxygen, the BMM solution was stored in a well sealed sterilised vial at 4°C (could be stored up to 2 weeks). If not used after preparing, bubble the solution with N_2 for a few minutes before use
Carbonate buffer	80 mM $NaHCO_3$, 120 mM Na_2CO_3 (pH 10.5)
SSC (20×)	3 M NaCl, 300 mM citrate
Proteinase K buffer	100 mM Tris (pH 8.0), 50 mM EDTA (pH 8.0) containing 1mg/ml proteinase K
In situ salts (10×)	3 M NaCl, 100 mM Tris pH8, 100 mM Phosphate buffer pH 6.8, 50 mM EDTA
Hybridization solution	1000 µl hybridization solution; 125 µl of 10 x in situ salts, 500 µl of deionized formamide, 250 µl of 50% dextran sulphate (warmed to aid pipetting before use), 25 µl of 50 x denhardt's solution (Sigma), 12.5 µl tRNA (100mg/ml) and 87.5 µl of sterilized nanopure water.
NTE buffer	0.5 M NaCl, 10 mM Tris pH 8.0, 1mM EDTA
Maleic acid buffer	100 mM Malic Acid, 150 mM NaCl, pH 7.5 adjusted with NaOH
Blocking reagent (10×)	10 gm blocking reagent (Roche Diagnostic) in 100 ml maleic acid buffer
Blocking solution	1ml of blocking solution: 100 µl of 10 x blocking reagent was added to 900 µl of Maleic acid buffer
TST	100 mM Tris pH 7.5, 150 mM NaCl, 0.3% Tween 20
Detection buffer	0.1M tris pH 9.5, 0.1M NaCl, 0.05M $MgCl_2$, sterilized through filter
NTMT buffer	100 mM NaCl, 100 mM Tris-HCl pH 9.5, 50 mM MgCl, 0.1% Tween 20
NBT/BCIP staining solution	NBT 4.5 µl ml^{-1}, BCIP 3.5 µl ml^{-1} of NTMT buffer
Fast Red staining solution	1 Fast Red tablet (Roche; 1 tablet contains 0.5 mg naphthol substrate, 2 mg Fast Red chromogen and 0.4 mg levamisole) dissolved in 2 ml of 100 mM Tris pH 8.2.

overnight) in EtOH/DTT: BMM (1:3 v/v), 2-4 hours (or overnight) in BMM and repetition of the overnight infiltration in freshly prepared BMM. Glass vials are required for infiltration. After infiltration, tissues were placed in a BEEM® capsule (ProSciTech) and capsules were filled to the top with freshly prepared BMM solution (Fig. 3.2). The lid of the capsule was closed so as to avoid air which impairs BMM polymerization. Capsules were organized in a grid made of wires (Fig. 3.2) and positions of capsules were marked along the wooden edges of the grid. Note that the capsules had to be separated from each other and were not labelled as writing on the capsules could interrupt the UV light pathway and affect polymerization. A cabinet which could fit in the –20°C freezer, with a reflecting inner surface (metal or foil paper), and containing a 6W UV lamp situated at the bottom of the cabinet at 25 cm from the capsules (Fig. 3.2) was used to polymerise samples. BEEM® capsules containing the tissues were hung in a "grid" (Fig. 3.2) to allow UV light and its reflection to reach all capsule surfaces. Polymerization of the samples was done at –20°C for 3-5 days and the status of polymerization was determined by testing the rigidity of the block using a needle. The capsules were removed and blocks were stored at 4°C until sectioning.

3.2.4 Sectioning

Blocks were trimmed to fit the specimen holder of the microtome. Trimming was done so as to orientate the tissue for either cross sections or longitudinal sections of the tissue. Sections of 5-8 μm thickness were obtained with a rotary microtome (Model 2055; Leica, Germany) using a glass knife and were collected individually using Tweezers 015 (ProSciTech). Sections were collected serially and were floated onto drops of sterilized water (6-8 μl) lying in rows on a clean, silane coated microscope

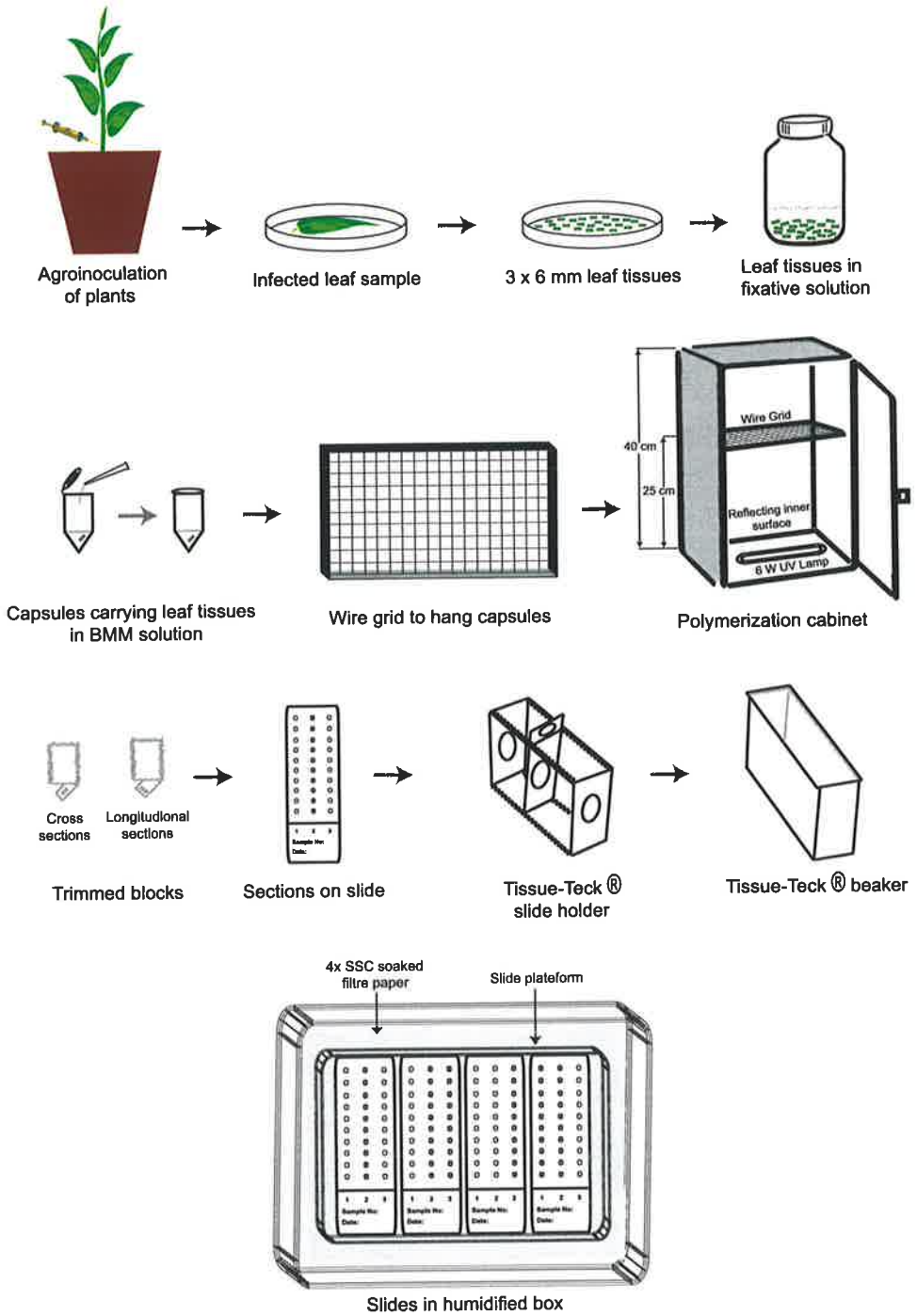


Figure 3.2. A diagrammatic description of the various steps of in situ hybridization. Detail for each step is described in the relevant section of Materials and Methods.

slide (ProSciTech). Slides were dried on a 42°C RNase free hot plate. Slides were labelled with pencil and were stored at 4°C before the pre-hybridization step.

3.2.5 Preparation of nonradioactive probes

A DNA template required to generate a nonradioactive probe was amplified by the polymerase chain reaction (PCR) using the primers specific to different geminiviruses and host factors (Rasheed et al., 2006; Selth et al., 2005). The amplified DNA was ligated into pGEM T Easy (Promega, Madison, WI), and the orientation of each insert was confirmed by sequencing. The constructs containing inserts in both sense and antisense orientation were selected to yield transcripts in both orientations using T7 DNA-dependent RNA polymerase (Promega). The template DNA to be used for in vitro transcription was purified, completely digested and free of RNase contamination. The plasmids were linearized with restriction enzymes chosen not to leave a 3' overhang. The linearized template DNA was treated with proteinase K (100 µg/ml proteinase K, 0.5% SDS, incubated at 50°C for 30 minutes) and subsequently purified by phenol-chloroform extraction (Sambrook & Russell, 2001). For a 20 µl in-vitro transcription reaction, 9µl of DNA template (1 µg DNA), 1 µl of RNAsin (40 u/µl; Promega), 2 µl of DIG-RNA labelling mix or fluorescence RNA labelling mix (Roche Diagnostics, Castle Hill, Australia), 4 µl of 5× transcription buffer (Promega), 2 µl of 100mM DTT (Promega), and 2 µl of T7 RNA Polymerase (Promega) were used. The reaction mixture was incubated for 2 hours at 37°C. To remove DNA from the transcripts, 2 µl RNAse free DNAse 1 (10 U/µl, Roche Diagnostics) was added and incubated for 15 min at 37°C. 1µl tRNA (10 mg/ml) and 80 µl sterilized nanopure water (SNW) was added and transcripts were precipitated with an equal vol (100 µl) of 4 M NH_4 acetate and 2.5 vol (250 µl) of ethanol at –80°C for 30 minutes. The reaction tube was centrifuged at

16,000 *g* for 15 min at 4°C to pellet the transcripts and the pellet was washed with 70% ethanol. Transcripts were resuspended in 100 µl sterilized nanopure water.

It was found that a probe of 200-400 bp size gave better results than a 1-2 kb probe. Transcripts were therefore hydrolysed in 100 µl carbonate buffer (Table 3.1) by incubating at 60°C. The optimal time (*t*) for carbonate hydrolysis was calculated to generate probe lengths of ~250-300bp using the formula: $t = (L_i - L_f) / (K \times L_i \times L_f)$, where t = time in min, L_i =initial length of probe in kb, L_f = final length of probe in kb, K is rate constant of 0.11kb/min.The reaction mixture was neutralised with 10 µl 10% acetic acid and probes were precipitated with 20 µl 3M Na Acetate, 500 µl ethanol at –80°C for 30 min. Probes were pelleted by centrifugation at 16,000 *g* for 15 min at 4°C and subsequently were washed with 70% ethanol, spun down for 5 min and dried. Probes were resuspended in 50 µl of 50% deionized formamide and were stored at –80°C. Eppendorf tubes containing fluorescein-labelled probes were wrapped with aluminium foil for longer durability.

3.2.6 Pre-hybridization treatments and washings

Pre-hybridization and post-hybridization was done in a Tissue-Tek® staining dish (ProSciTech), which could accommodate 200 ml of buffer and a supplied Tissue-Tek® slide holder (ProSciTech). The methacrylate embedding medium around the sections was removed by dipping the slides in an acetone series as follows: 100% acetone for 15 min, 100% acetone for 10 min, 50% aqueous acetone for 5 min and sterilized nanopure water for 5 min (2 washings). Slides were transferred to 2× SSC (Table 3.1) for 15 min and treated with proteinase K buffer (Table 3.1) for 30 min at 37°C. The proteinase K digestion was stopped by incubating the slides in 0.2% glycine in PBS for 3-5 min at

room temperature and washing twice with PBS for 2 min. Slides were treated with freshly prepared 4% paraformaldehyde (4% paraformaldehyde in PBS containing 1mMDTT without 0.25% glutaraldehyde) in the fume hood for 10 min and washed twice in PBS for 5 min each. Slides were equilibrated with 0.1M triethanolamine and acetic anhydride (12.5 ml 2M triethanolamine, 237.5 ml sterilized water and 2.5 ml acetic anhydride) for 10 min with agitation at room temperature. The slides were washed twice with PBS for 5 min each before dehydration in a graded series of aqueous 30%-100% ethanol for 30 sec each. The slides were air dried in a laminar flow chamber and stored in a Tissue-Tek® beaker with a little 100% EtOH in the bottom (not touching the samples) at 4°C until hybridization.

3.2.7 Hybridization

The hybridization solution (Table 3.1) was prepared allowing 125 µl per slide. The hybridization solution was warmed to 65°C and mixed gently without forming bubbles. Two microliters of probe were diluted to 25 µl in 50% deionized formamide and denatured at 80°C for 2 minutes. A range of dilutions was tested to find the optimal amount. The diluted probe was gently applied to the slide and spread evenly over the section sample. Gloves were changed each time a new probe was used. The slides were covered with Hybri-slips® (Sigma) and were placed in a humidified box (3MM filter paper soaked in 4x SSC) and incubated at 42°C overnight (Fig. 3.2).

3.2.8 Post hybridization treatments and washings

After hybridization, Hybri-slips® were removed by dipping the slides into a beaker containing 2× SSC at 55°C. The Hybri-slips® were slid off and slides were treated with 0.2× SSC at 55°C for 45 min with agitation. This treatment was repeated twice with

fresh 0.2× SSC for 45 minutes each. The slides were equilibrated twice with NTE buffer (Table 3.1) at 37°C for 5 min each. The slides were placed in fresh NTE containing 20 µg/ml RNase A at 37°C for 30 min to remove excess and unbound probes from the tissue. They were washed twice with NTE at 37°C for 5 min each. To increase the stringency of washing, the slides were placed in 0.2× SSC containing 0.3% Triton X-100 at 55°C for 60 min with gentle agitation. At this stage slides could be stored at 4°C overnight in a beaker of PBS sealed with parafilm.

3.2.9 Blocking and incubation with antibody

The slides were placed flat on an elevated platform in the humid box and were blocked with 300 µl filtered (Millipore filter, 0.2µm) blocking solution (Table 3.1). The slides were incubated for 30 min at room temperature. The blocking solution was removed and 300 µl filtered 1% BSA in TST (Table 3.1) was applied and slides were again incubated for 30 min at room temperature. This was replaced with 300 µl anti-DIG fab alkaline phosphatase conjugate or anti-fluorescein alkaline phosphatase conjugate (Roche Diagnostics) diluted 1:300 in filtered 1% BSA TST. The slides were incubated for 1 h, and then washed 3 times for 10 minutes each in a beaker containing TST with agitation at room temperature.

3.2.10 Colour Reaction

Slides were placed in detection buffer (Table 1) for 10 minutes (no agitation) to equilibrate the pH to 9.4-9.5. Slides were placed flat and 300 µl NBT/BCIP or Fast Red staining solution (Table 3.1) was added to each slide. The slides were covered with Hybri-slips® and placed in a darkened humid box. This step was done quickly to avoid non-specific colour production in the tissue. Colour development was checked after 4-6

h under a microscope. It was continued for up to 40 h but for viral DNA 6 h was generally sufficient. It was important not to let the slides dry. To stop the reaction the slips were removed in a beaker containing TE buffer (Table 3.1) and the slides were then placed in water. The sections were dehydrated in an aqueous ethanol series (30% to 100%) for 10 sec per treatment. This treatment was rapid because the blue coloured product is "slightly" soluble in ethanol. Slides were air dried in a dust free environment and mounted in Entellan mounting medium (ProSciTech).

3.2.11 Double labelling

Dual colour in situ hybridization was done to target two different DNA sequences in the same cell. Probes designed to target the specific sequences were labelled with DIG or fluorescein (see section 2.6) and were applied simultaneously. The probes were detected sequentially using NBT/BCIP substrate for DIG-labelled probes and Fast Red substrate for fluorescein-labelled probe. In general, fluorescein-labelled probes are less stable than DIG-labelled probes and therefore they were visualized first. To detect fluorescein-labelled probe, the slides were treated with anti-fluorescein antibody conjugated with alkaline phosphatase (AP) and stained with Fast Red substrate as mentioned above in section 2.2.9 and 2.2.10. After detecting the fluorescein-labelled probe, AP enzyme was inactivated by incubating the slides in 100 mM glycine-HCl, pH 2.2 for 30 min. The slides were washed three times with 1× PBS and transferred to anti-DIG antibody treatment and NBT/BCIP staining as explained above.

3.2.12 RNase treatment and hybridization under denaturing and non-denaturing conditions

For RNase treatment, the in situ hybridization protocol was modified as follows: the slides were incubated in 1× TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA) containing 20 μg ml^{-1} DNase-free RNase-A for 50 min at 37°C, followed by two washes in TE buffer. Proteinase treatment was done using 100 mM Tris-HCl (pH 8.0), 50 mM EDTA containing 1 mg ml^{-1} proteinase K. To inactivate any residual RNase A, slides were placed in PBS buffer (0.13 M NaCl, 0.007 M Na_2HPO_4, 0.003M Na_2HPO_4, pH 7.0) containing 0.1% diethyl pyrocarbonate (DEPC) for 20 min. To hydrolyze DEPC, the slides were washed with PBS and then incubated in 1× NTE (500mM NaCl, 10 mM Tris-HCl pH 8.0, 1 mM EDTA) two times for 15 min each prior to hybridization.

To differentiate ssDNA from dsDNA of geminiviruses, in situ hybridizations were done under both denaturing and non-denaturing conditions. To denature target DNA in tissue sections, formamide hybridization buffer containing probe mixture was applied to slides. Slides were covered with Hybri-slips® and placed on a hot plate at 80°C for 4 min prior to hybridization at 42°C. If air bubbles were produced during the heat treatment they were not removed by pressing the cover slips because of the risk of damage to sections. Non-denaturing hybridization was done at 42°C without prior heat treatment of the slides.

3.2.13 Microscopy and data processing

Sections were analysed with a Zeiss Axioplan microscope (Carl Zeiss, Jena, Germany) using differential interference contrast (DIC) optics and SPOT software. The digitized

images were further processed with Adobe Photoshop software (Adobe systems, Inc., San Jose, CA).

3.3 Results

3.3.1 Fixation, embedding and sectioning

This study showed that a combination of paraformaldehyde (4%) with glutaraldehyde (0.25%) gave better fixation than paraformaldehyde fixation. To ensure complete infiltration of the fixative solution, tissue samples in fixative solution were subjected to low pressure infiltration prior to overnight fixation at 4°C (see section 3.2.2). However, it is important to release the vacuum slowly to avoid damage to the morphology of tissue. The fixation should be performed in a glass vial of reasonable size with an ample amount of fixative solution (~5 ml) because the ratio of tissue to fixative solution (1:10) is important for complete fixation (Fig. 3.2). Complete dehydration with a series of ethanol after fixation is essential to maintain the morphology of the plant tissue.

An appropriate combination of methyl methacrylate and butyl methacrylate (BMM; Table 3.1) and complete infiltration of BMM embedding solution into tissues was important for obtaining sections of different thicknesses (5-40 μM) (Fig. 3.3C and 3.3D). Poor embedding results in crumbled sections (Fig. 3.3E). Thinner sections (5-10 μM) were found to give better results compared to thicker sections (> 40 μM) as thicker sections has produced shading effect which can be confused with the dark blue staining of NBT/BCIP. This becomes more important when observing sections of leaf veins because thick xylem vessels produce a dark shading effect (compare Fig. 3.3 D with 3.3C and 3.3A). Another problem with thicker sections was that the rate of loss of those sections during pre- and post-hybridization washings was very high (up to 90%)

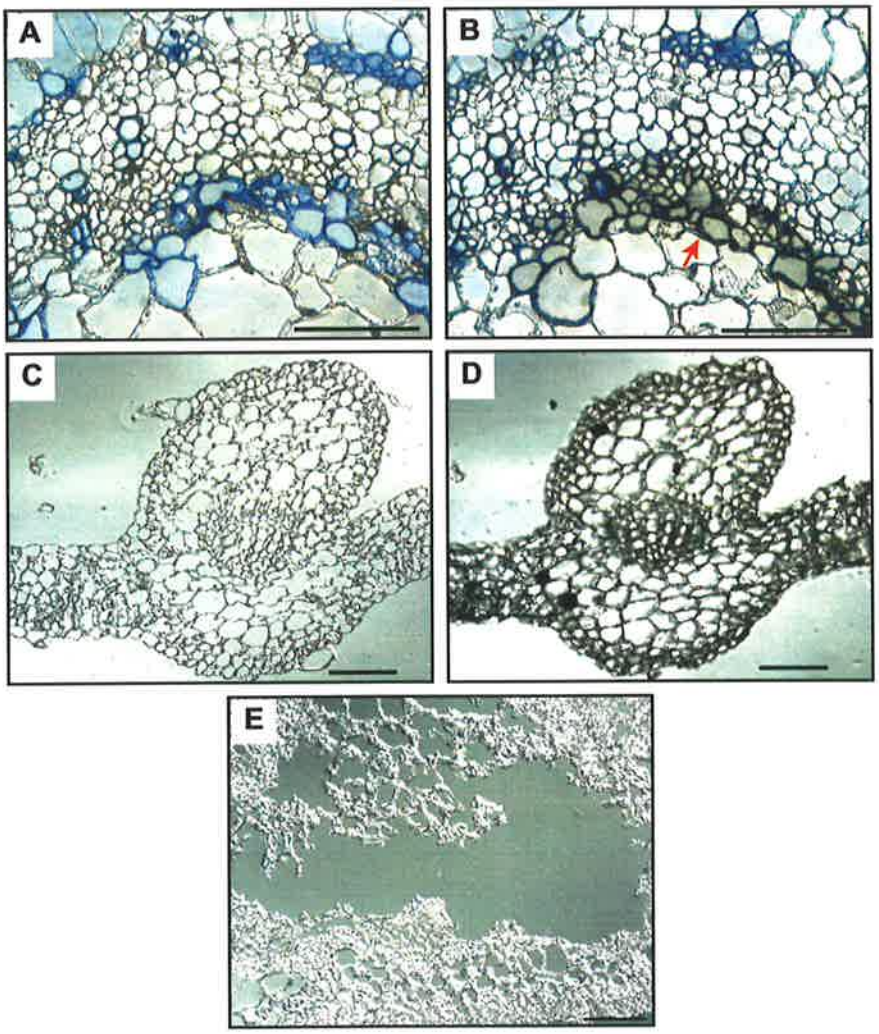


Figure 3.3. Examples of staining and sectioning problems. Transverse sections of leaf vein of *N. benthamiana* (A-E). A TLCV specific probe was hybridized with sections taken from TLCV-infected plant (A and B). **(A)** A section equilibrated with detection buffer and stained with NBT/BCIP staining solution pH 9.5. **(B)** A section stained with NBT/BCIP staining solution of low pH (< 9) without prior detection buffer treatment. Red arrow indicates the dark staining spot. **(C)** a section of 5 μM thickness and **(D)** a section of 40 μM thickness. **(E)** An example of incomplete fixation. Bars = 100 μm.

3.3.2 RNA probes

The size of RNA probes confirmed by analysing $1/100^{th}$ of each of the transcripts by agarose formaldehyde electrophoresis (Sambrook & Russell, 2001) before the hydrolysis step. A sharp band of RNA of appropriate size was clearly visible (Fig. 3.4A). The yield of probe was estimated by spectrophometry. However, the accurate concentration and labelling efficiency of the probe (labelled with DIG or fluorescein) was determined by running the different concentrations of the probe on a 1% formaldehyde RNA gel and transferring to Zeta-Probe membrane (BioRad, Hercules, CA) following the AP conjugated DIG or fluorescein-labelled antibody treatment and subsequently detected by AP substrate (Fig. 3.4B) (see section 4.2.4, chapter 4). For ISH, different dilutions of the probe were tested. A dilution of 1:75 to 1:100 of the original probe preparation was found to be optimal during all ISH experiments (Fig 3.5).

3.3.3 RNase treatment and hybridization of sections under denaturing and non-denaturing conditions

Geminiviruses contain ssDNA and replicate in the nuclei of infected plant cells through dsDNA. Infected plant cells contain ssDNA, dsDNA and RNA transcripts of both polarities (see Fig 1.9) and a probe designed to detect ssDNA also has the potential to hybridize with virion sense RNA transcripts (see Fig 4.1, Table 4.1). Enzymatic removal of transcripts by treating the tissue with DNase free RNase A before hybridization (see section 3.2.12) is a critical step because contamination with RNase A can degrade RNA probes. Therefore, after RNase treatment, its inactivation (see section 3.2.12) was necessary for successful ISH. A RNA probe, designed to detect ubiquitin transcripts, produced strong chromogenic signals in the cells of section without RNase

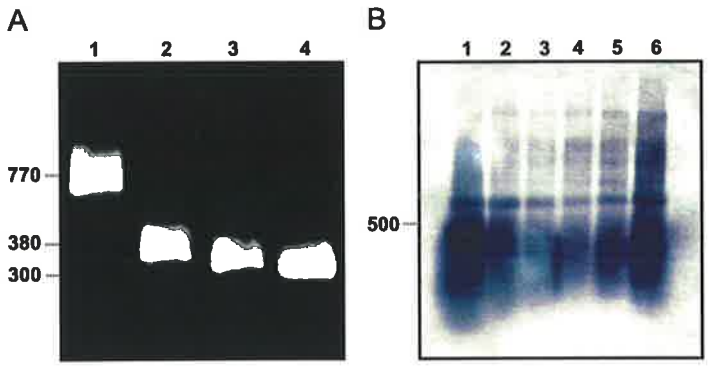


Figure 3.4. Detection and quantification of nonradioactive RNA probes. **(A)** Showing RNA probes of different sizes (lane 1-4). 1 µl of in vitro transcript product was loaded on a 1% agarose gel containing formaldehyde. **(B)** RNA probe of 771 nt (shown in A, lane 1) in sense (lane 1-3) and antisense (lane 4-6) orientations was hydrolyzed, electrophoresed in a 1% gel containing formaldehyde and transferred to the membrane for detection of enzyme (AP) activity. Lane 1 and 6 contained 2 µl; lane 2 and 5, 0.5 µl and lane 3 and 4, 0.2 µl of the hydrolyzed probe.

treatment (Fig. 3.5C). However, signals were not detected with the same probe in RNase treated sections (Fig. 3.5D) showing that the RNase treatment was effective in removing the ubiquitin transcripts from the cell.

dsDNA can be detected by denaturation prior to hybridization (see section 3.2.12). A significant increase in the intensity of hybridization signals was found following heat denaturation of sections (Fig. 3.5B) compared to non-denatured sections (Fig. 3.5A) using TLCV-specific probes designed to detect dsDNA (Rasheed et al., 2006) (chapter 4).

3.3.4 Single and double staining

Good quality staining requires accurate pH and proper composition of staining buffer. For DIG-labelled probe, a bright blue colour with uniform staining was obtained using NBT/BCIP in NTMT buffer at pH 9.5 (Fig. 3.3A). It was found to be important to equilibrate the slides with detection buffer (Table 3.1) before applying staining solution to the slides. Uneven staining with dark spots was obtained when staining solution of low pH (<9) was applied to the slide which was not previously equilibrated with detection buffer (Fig. 3.3B, red arrow).

As an example of single and double labelling, it was demonstrated that the expression of a tomato protein from NAC family, *SlNAC1*, was enhanced in TLCV-infected cells (Selth et al., 2005). A tissue section taken from TLCV-infected plant was hybridized simultaneously with SlNAC1 specific fluorescein-labelled probe and TLCV-specific DIG-labelled probe. Both probes were detected sequentially; first fluorescein-labelled probe and then DIG-labelled probe. Bright red signals were obtained when fluorescein-

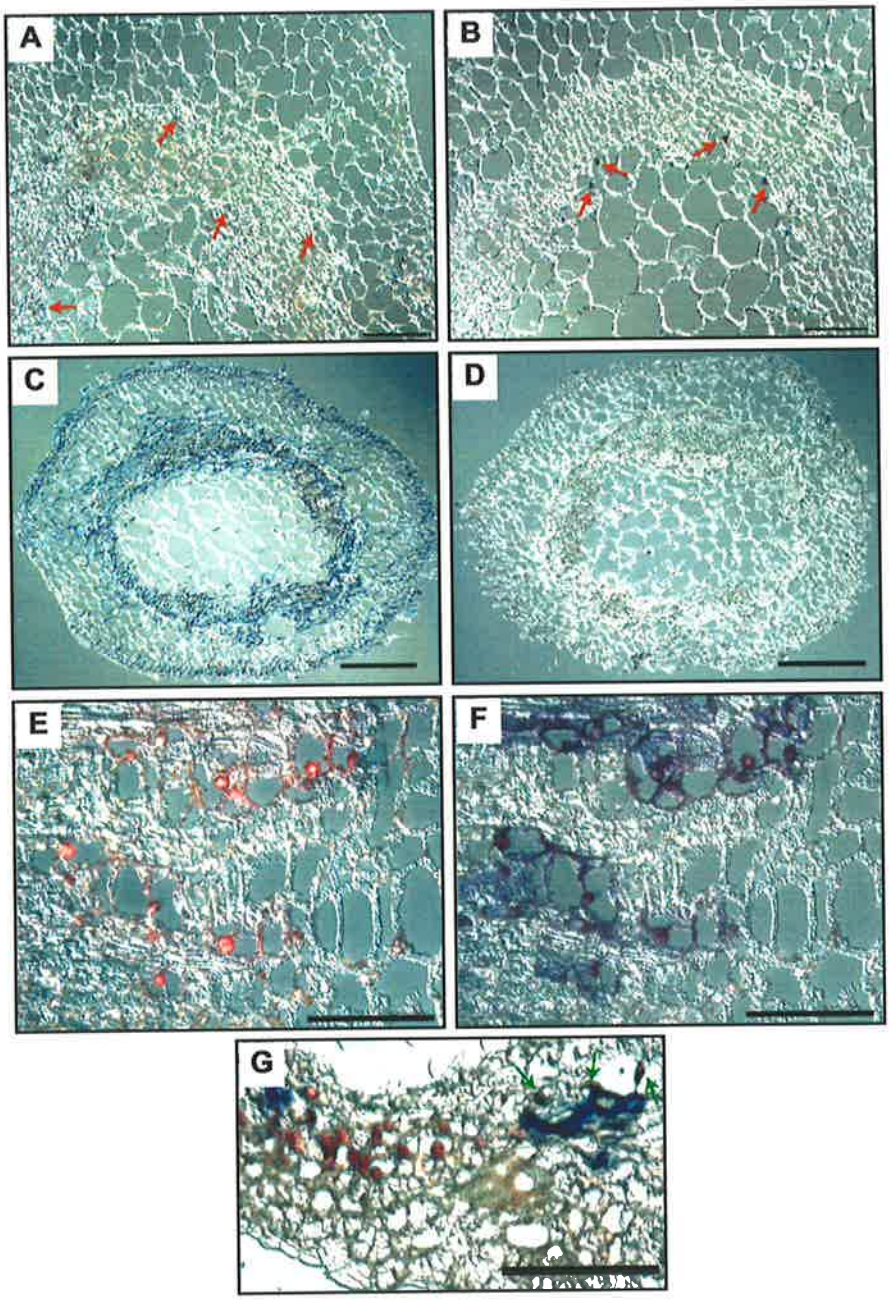


Figure 3.5. Examples of in situ hybridization. **(A-B)** Denaturing treatment, the section was heated to 80°C to denature viral dsDNA prior to hybridization as described in the methods. Stronger hybridization signals were detected in the nuclei of denatured section (B) than non-denatured section (A). Red arrows indicate the signals of viral dsDNA in nuclei. **(C-D)** RNase treatment, the section was treated with RNase A prior to hybridization to remove ubiquitin transcripts (D). Ubiquitin transcripts were detected in the section without RNase A treatment (C). **(E-G)** Double labeling, Induction of *SlNAC1* by TLCV was dected by hybridization with fluorescein-labelled ssRNA *SlNAC1* specific probe (E) and presence of TLCV in the same section was confirmed by hybridization with digoxigenin-labelled TLCV specific probe (F). Detection of TLCV (blue) and TGMV (red) in the same section (G) taken from co-infected plant was done by hybridization with digoxigenin-labelled TLCV probe and fluorescein-labelled TGMV-specific probe. Green arrows in G indicate the cells where the red colour was not completely masked by the blue colour. Bars = 100 μm.

labelled probe was detected by staining with fast red (Fig 3.5E). However, the red signals were masked by blue colour to produce purple colour when same section was treated with NBT/BCIP staining solution to detect DIG-labelled probe (Fig. 3.5F). Another example of double labelling, is the co-localization of two distinct geminiviruses, monopartite TLCV and bipartite TGMV, in mixed infection (Rasheed et al., 2006); chapter 4). It has been reported that TLCV ssDNA accumulates in the cytoplasm of phloem cells and contrary to that TGMV ssDNA is found in the nuclei of both vascular and non-vascular cells (Rasheed et al., 2006); chapter 4). Strong blue signals were observed in the cytoplasm and nucleus of vascular cells using TLCV-specific DIG-labelled probe (Fig. 3.5G) and bright red signals were detected in the nuclei of mesophyll cells using TGMV-specific fluorescein-labelled probe (Fig. 3.5G). TGMV-specific red signals in the vascular cells were completely masked by TLCV-specific blue signals. However, some purple signals, where the intensity of blue signals was less were also evident as indicated by green arrows (Fig 3.5G). It was noticed that the intensity of red signals was less than the blue signals (Fig. 3.5E, 3.5F and 3.5G).

3.4 Discussion

The interpretation of ISH results relies on the inclusion of appropriate positive and negative controls in each experiment. The specificity of targeted nucleic acid detection should be confirmed by several tests: (i) comparing sense probe as a control with anti-sense probe for the detection of mRNA, (ii) inclusion of tissue sections from uninfected plants in the experiments for the detection of viruses, and (iii) using a non-specific probe to target nucleic acid or omitting the probe from hybridization mix. Hybridization signals should not be detected in negative control tests. Controls are essential when performing dual colour ISH experiments. It is important to do independent ISH

experiments with differentially labelled probes on separate sections before performing dual colour ISH on one section.

Proper fixation and embedding are crucial steps for a successful ISH. Fixation is required to maintain the morphology of tissue and to protect the nucleic acids within the cells. It has been observed that penetration of aldehyde fixatives to plant tissues is rapid but the rate of penetration decreases with time (de Almeida Engler et al., 2001). The role of embedding is to allow preparation of sections of appropriate thickness with intact morphology and cellular structure. Wax embedding using paraffin is the most convenient way of embedding. However, high resolution and structural preservation can be obtained by embedding the tissues in a dissolvable plastic medium consisting of a mixture of methyl methacrylate and butyl methacrylate (Gubler, 1989; Kronenberger et al., 1993).

While fixation is essential for the efficient retention of nucleic acid and preservation of cellular structure, overfixation can decrease the accessibility of the probe to the targeted nucleic acid. Permeabilization of the tissue with proteinase K treatment enhances the sensitivity of the technique (Jowett, 2001). Furthermore it was observed that proteinase K treatment significantly enhanced the signal intensity. It is generally accepted that probes of shorter lengths more readily penetrate fixed tissues. Therefore, hydrolysis of the RNA probes has been recommended (Angerer et al., 1987). In this study it was found that long probes (up to 1kb) gave satisfactory results.

High activity and sequence specificity of probes are important factors for the success of ISH. To enhance the sensitivity of the hybridization, it is important to purify the probes

from non-incorporated nucleotides. Precipitation of probe with 0.5 vol 4M NH_4 acetate (pH 8.0) and 3 vol ethanol is reported to remove free nucleotides (Hauptmann & Gerster, 1994). Removal of DNA contaminants from the probe transcripts by RNase free DNase-I greatly reduces the background staining. If a background problem persists, RNase digestion in high salt to remove unbound probe may help to reduce the background. It is essential to completely digest the plasmid DNA templates, since a small amount of supercoiled uncut plasmid will result in the generation of long transcripts containing the plasmid sequences which will result in increased background. It has been suggested that the transcription of templates with protruding 3' termini can result in the synthesis of complementary strand RNA molecules that are aberrantly initiated at the protruding 3' termini of the templates (Schenborn & Mierendorf, 1985). Therefore, it is recommended to avoid digestion which will leave 3' overhang.

Although DNA probes are commonly used for ISH, the technique described in this study is based on the use of RNA probes. RNA probes were selected because RNA-RNA and RNA-DNA duplexes are more stable than DNA-DNA duplexes (Casey & Davidson, 1977) and therefore more stringent washings can be performed to remove unbound probes that can reduce background staining and improve the sensitivity. The efficiency of hybridization and detection of probe depends on various parameters, for example, type (DNA vs RNA and double- vs single-stranded probe), size (shorter probes have higher penetration rate and hybridization interaction), specificity (complementarity to the target), activity (labelling efficiency) and concentration (in relation to target saturation) of probe (Abraham, 2001). Hybridization conditions and pre- and post-hybridization washings significantly affect the sensitivity of the ISH.

The modified ISH procedure described above has been used in this thesis to detect various forms of viral nucleic acids and host transcripts (chapters 4, 5, 6 and 7) and to study host-pathogen interactions at the cellular level.

Chapter 4 – Single-stranded DNA of *Tomato leaf curl virus* accumulates in the cytoplasm of phloem cells

4.1 Introduction

Plant virus infection requires successful invasion of host tissues. Systemic spread from the primary site of infection initially involves cell-to-cell movement through plasmodesmata (Pd) and then distal movement via the vasculature (Carrington et al., 1996). Plant viruses are classified as phloem-limited if they are restricted to the vascular system or mesophyll-invasive if they infect non-vascular cells.

Vascular bundles of dicotyledonous species comprise xylem, phloem parenchyma, cambium, companion cells and sieve elements (Nelson & van Bel, 1998). If a mesophyll-invasive virus first infects an epidermal cell, virus trafficking to and within vascular bundles requires movement from mesophyll cells to phloem parenchyma and sieve elements (Nelson & van Bel, 1998). To exit the vasculature, this sequence is thought to be reversed. In contrast, phloem-limited viruses must enter the vascular tissues directly to establish a systemic infection. It is not understood at the molecular level how viruses enter, travel through, or depart the vascular system. It has been suggested that the Pd between vascular cells differ from those connecting mesophyll cells (Nelson & van Bel, 1998), and this is supported by the observation that certain cell types within the vascular system are more easily infected than others (Ding et al., 1995).

The reasons why some viruses are restricted to vasculature while others are capable of infecting the mesophyll remain unclear. It has been suggested that vascular-restricted

infection might occur because viral movement proteins (MPs) are unable to function in epidermal and mesophyll cells (Taliansky & Barker, 1999). Another hypothesis is that some viruses cannot suppress host defence responses in non-vascular cells (Voinnet et al., 1999; Waterhouse et al., 1999). The observation that vascular limitation of phloem-restricted viruses can be overcome by co-infection with mesophyll-invasive viruses supports this latter idea (Morra & Petty, 2000; Wege et al., 2001). Tissue specificity of a virus can also be affected by environmental factors (Ding et al., 1999b) and the developmental stage of the plant (Wang et al., 1996).

All previous studies of geminiviral DNA localization have indicated that it accumulates exclusively in the nucleus of infected cells. For example, TGMV infection led to the accumulation of virions, often as paracrystalline arrays, in nuclei of both vascular and non-vascular cells of *N. benthamiana* (Rushing et al., 1987). Bass et al. (2000) observed altered nuclear architecture and plant chromatin condensation upon TGMV infection, indicating that viral DNA was present in the nucleus. Moreover, studies of the tissue specificity of both monopartite (Morilla et al., 2004; Rojas et al., 2001) and bipartite (Morra & Petty, 2000; Qin & Petty, 2001; Wege et al., 2001) geminiviruses by in situ hybridization showed that viral DNA accumulated in the nuclei of infected cells.

It has been suggested that geminiviruses move from cell-to-cell and systemically either in the form of virions or in nucleoprotein complexes (Gafni & Bernard, 2002). However, the form of viral DNA involved in viral trafficking is unknown. The NSP and MP of BDMV, a mesophyll-invasive bipartite begomovirus, bind ss- and ds-DNA in a form- and size-selective manner (Rojas et al., 1998), and dsDNA is the predominant form of viral cargo (Noueiry et al., 1994; Rojas et al., 1998). In contrast, the NSP from

SqLCV, a phloem-limited bipartite begomovirus, binds strongly to ssDNA but weakly to dsDNA, while the MP associates weakly with ssDNA and does not appear to bind dsDNA at all (Pascal et al., 1994). A phage gene 5 protein (M13; g5p) which binds viral ssDNA inhibited the movement of *Tomato leaf curl New Delhi virus*- (ToLCNDV), suggesting that ssDNA moves cell-to-cell in ToLCNDV infection (Padidam et al., 1999). It has been proposed that different tissue tropisms exhibited by bipartite geminiviruses might be the result of different movement mechanisms (Morra & Petty, 2000).

This study describes the intracellular localization of ssDNA and dsDNA of the monopartite begomovirus TLCV in comparison with that of the bipartite begomovirus, TGMV, in both single- and mixed-infections. These in situ hybridization studies have shown that ssDNA plays a major role in TLCV trafficking through the cytoplasm.

4.2 Materials and Methods

4.2.1 Plant material and inoculation

Nicotiana benthamiana, tobacco (*N. tabacum* cv. Samsun) and tomato (*Solanum lycopersicum* L. cv. Grosse Lisse) were maintained at 25-30°C with a 16 h photoperiod under containment conditions. Plants at the 4-5 leaf stage were inoculated with infectious viral clones (Table 4.1) using *Agrobacterium tumefaciens* (Grimsley et al., 1987). Young (3^{rd}), developing (7^{th}) and mature (12^{th}) leaves, stems, petioles and roots were sampled at 3-4 weeks post-inoculation and processed for fixation as described below. Infection was monitored by dot blot analysis of viral DNA using ^{32}P-labelled complementary DNA probes (Stonor et al., 2003).

Table 4.1. Infectious clones of TLCV, TGMV and TYLCSV DNA

Plasmid Designation	Construct	Source
pBin TLCV wt	2.0 mer	Dry et al. (1993)
pBin TGMV A	2.0 mer	von Arnim and Stanley (1992)
pBin TGMV B	2.0 mer	von Arnim and Stanley (1992)
pBin TYLCSV wt	2.0 mer	Kheyr-Pour et al. (1991)

4.2.2 Tissue processing

Tissues were cut into 4 mm by 6 mm pieces and fixed in 4% paraformaldehyde: 0.25% glutaraldehyde and were embedded in buty-methyl methacrylate (chapter 3). Sections 8 µm in thickness were obtained with a rotary microtome (Model 2055; Leica, Germany) and placed on silane-coated slides (ProSciTech, Thuringowa Central, Australia).

4.2.3 Preparation of RNA probes

DNA fragments were amplified by the polymerase chain reaction (PCR) using specific primers (Table 2.2). Probes were labelled with either digoxigenin (DIG)-11-dUTP or fluorescein-12-dUTP using DIG or Fluorescein RNA Labelling Mixes (Roche Diagnostics, Castle Hill, Australia) by *in vitro* transcription as described in section 3.2.5.

4.2.4 DNA gel blot analysis using non-radioactive probes

Total nucleic acid was extracted as described previously (Dry et al., 1993) from TLCV and TGMV infected *N. benthamiana* and tomato plants. The DNA was electrophoresed in a 1.2% agarose gel, blotted onto Zeta-Probe membrane (BioRad, Hercules, CA) (Dry et al., 1993) and hybridized overnight at 42°C with strand-specific DIG-labelled TLCV- and TGMV-specific probes (Fig 4.1A; Table 4.2). Membranes were washed with 2 × SSC (1 × SSC = 0.15 M NaCl and 0.015 M trisodium citrate) twice for 5 min each and then once for 30 min with 0.2 × SSC containing 0.3% Triton-X100. The membranes were blocked in 1 × blocking solution (Roche Diagnostics) for 30 min, soaked in TST buffer (100 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.3% Tween-20) containing 1% bovine albumin serum (BSA) for 30 min, incubated in 1% BSA TST buffer containing anti-DIG alkaline phosphatase (AP; 1:5000) for 30 min, washed twice in TST buffer for

Table 4.2. TLCV, TGMV and TYLCSV specific ribonucleotide probes

Probes[a]	Source	Nucleotide Position[b]	Size (nt)	Potential targets
VS-cp	TLCV	308-1078	771	dsDNA
CS-cp	TLCV	1078-308	771	ss and dsDNA, CP transcript
VS-rep	TLCV	2156-2464	309	dsDNA and Rep transcript
CS-rep	TLCV	2464-2156	309	ss and dsDNA
VS-ir	TLCV	2656-127	238	dsDNA
CS-ir	TLCV	127-2656	238	ss and dsDNA
VS-cp	TGMV	327-1070	744	dsDNA
CS-cp	TGMV	1070-327	744	ss and dsDNA, CP transcript
VS-rep	TGMV	1962-2210	249	dsDNA and Rep transcript
CS-rep	TGMV	2210-1962	249	ss and dsDNA
VS-rep	TYLCSV	2170-2466	309	dsDNA and Rep transcript
CS-rep	TYLCSV	2466-2170	309	ss and dsDNA
CS-ubi	Ubiquitin	-	-	Ubiquitin transcript

[a] VS, virion sense; CS, complementary sense; cp, coat protein; rep, replication protein; ir, intergenic region; ubi, ubiquitin

[b] Nucleotide position for TLCV as in Dry et al. (1993), for TGMV as in von Arnim and Stanley (1992) and for TYLCSV as in Kheyr-Pour et al. (1991)

10 min each, and then equilibrated in detection buffer (0.1 M Tris-HCl pH 9.5, 0.1 M NaCl, 0.05 M $MgCl_2$) for 5 min. AP activity was detected by incubating in NBT/BCIP staining solution (Table 3.1) for 30-60 min until a dark-bluish colour developed. The reaction was stopped with TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5).

4.2.5 In situ hybridization

Pre-hybridization, hybridization and post-hybridization were done as described in chapter 3. The DIG- or fluorescein-labelled probes were detected by anti-DIG or anti-fluorescein fab alkaline phosphatase conjugates (Roche Diagnostics) and subsequently stained by NBT/BCIP or Fast Red substrates (Roche Diagnostics). Dual colour in situ hybridization was done by applying the probes simultaneously and detecting them sequentially (chapter 3, section 3.2.11). For RNase treatment the in situ hybridization protocol was modified as explained in chapter 3 (section 3.2.12). To differentiate ssDNA from dsDNA, in situ hybridizations were done under both denaturing and non-denaturing conditions as described in chapter 3 (section 3.2.12).

Sections were photographed with a SPOT digital camera mounted on a Zeiss Axioplan microscope (Germany) using differential interference contrast (DIC) optics and SPOT software. The contrast and brightness of images were subsequently adjusted and processed using Adobe Photoshop software (Adobe Systems Inc., San Jose, CA).

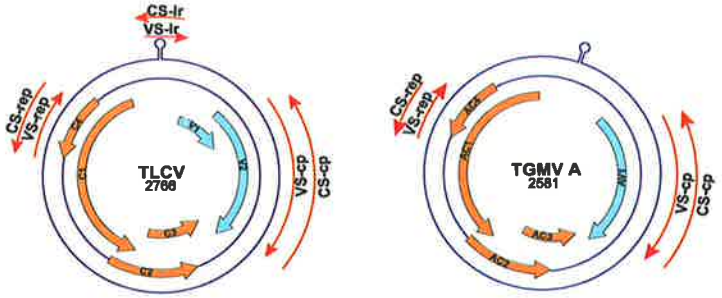
4.3 Results

4.3.1 Specificity of riboprobes to detect targeted viral nucleic acid forms

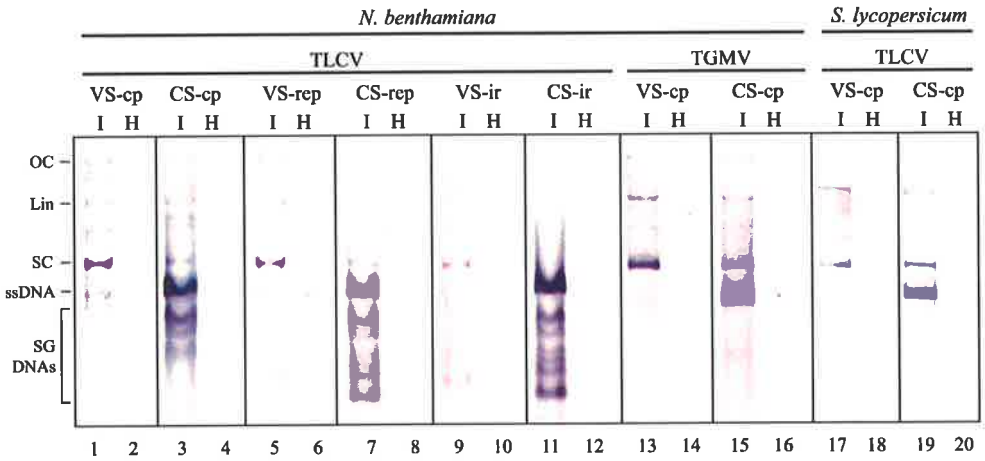
To identify the different viral DNA species at the subcellular level using in situ hybridization, a series of digoxigenin- (DIG) and fluorescein-labelled probes corresponding to different regions of TLCV and TGMV DNA (Fig. 4.1A and Table 4.2) was used. The ability of probes to differentiate single-stranded and double-stranded replicative form DNA was tested by DNA gel blot hybridization in a preliminary experiment. As shown in Fig. 4.1B, the probes hybridized with dsDNA replicative forms and ssDNA in a strand-specific manner. A larger amount of ssDNA was detected compared to other forms of viral DNA using complementary-sense probes specific for coat protein, replication associated protein and intergenic region (Fig. 4.1B, Table 4.2). It is worth noting that a small amount of DNA corresponding to complementary-sense (cs) ssDNA was also visible in lanes 1 and 5 using virion-sense probes (Fig. 4.1B). A relatively low level of cs ssDNA has also been reported in ACMV infection (Saunders et al., 1991). Furthermore, subgenomic DNAs were found in *N. benthamiana* plants agroinoculated with TLCV but not in tomato. In contrast, subgenomic DNAs were not found in *N. benthamiana* plants agroinoculated with TGMV (Fig. 4.1B).

The specificity of the probes was tested by hybridization to tissue sections from a mock-inoculated *N. benthamiana* plant. No signals were observed in the mock-inoculated sections (Fig. 4.1C1), indicating that non-specific binding to the sections was not occurring. The TGMV- or TLCV-specific probes designed to target viral DNA and RNA transcripts (CS-cp; Table 4.2) produced strong chromogenic signals in leaf sections infected with the respective viruses (Fig. 4.1C4 and 4.1C5). When diseased plants, which may have a different physiology from healthy plants, were hybridized

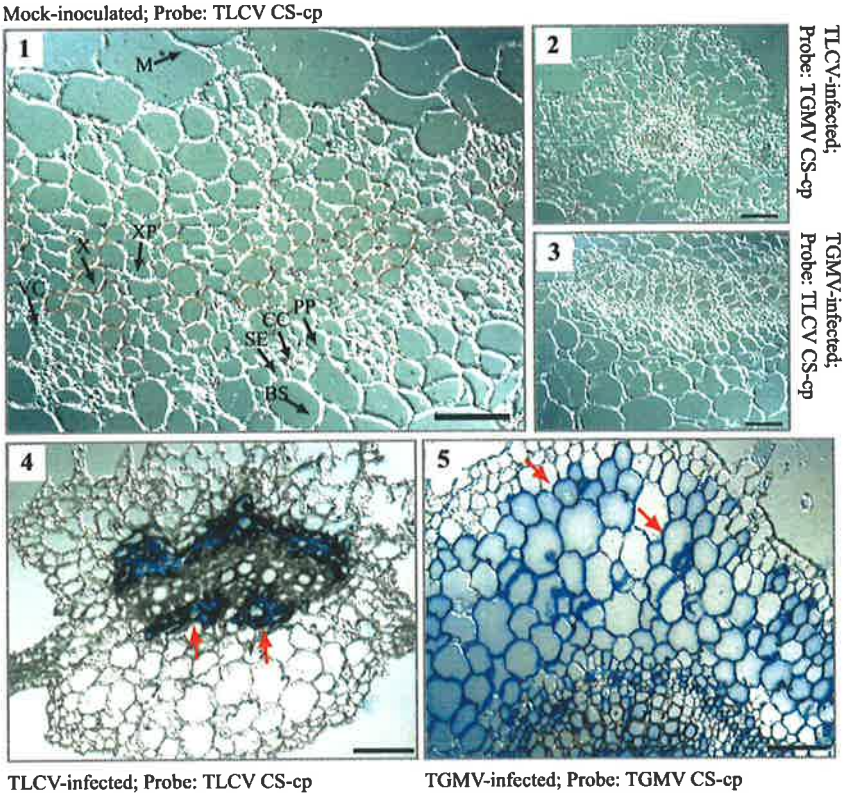
A


CS-ir
VS-ir
CS-rep
VS-rep
TLCV
2768
V1
V2
C1
C2
C3
C4
VS-cp
CS-cp
TGMV A
2581
AV1
AC1
AC2
AC3
AC4

B


N. benthamiana
S. lycopersicum
TLCV
TGMV
TLCV
VS-cp
CS-cp
VS-rep
CS-rep
VS-ir
CS-ir
VS-cp
CS-cp
VS-cp
CS-cp
I H I H I H I H I H I H I H I H I H I H
OC
Lin
SC
ssDNA
SG
DNAs
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

C


Mock-inoculated; Probe: TLCV CS-cp
1
M
X
XP
VC
CC
PP
SE
BS
2
TLCV-infected;
Probe: TGMV CS-cp
3
TGMV-infected;
Probe: TLCV CS-cp
4
5
TLCV-infected; Probe: TLCV CS-cp
TGMV-infected; Probe: TGMV CS-cp

with probes for the heterologous virus, no signals were observed. For example, sections of a TLCV-infected plant probed with TGMV-specific CS-cp RNA (Fig. 4.1C2) and a TGMV-infected plant hybridized with TLCV-specific CS-cp probe (Fig. 4.1C3) confirmed that the in situ hybridization procedure was specific for detecting homologous viral nucleic acids.

4.3.2 TLCV is a vascular associated virus

Using in situ hybridization, TLCV was found only in the vascular tissue of systemically infected *N. benthamiana* leaves (Fig. 4.1C4 and Fig. 4.2A-I). Infected cells were more abundant in young symptomatic leaves than in developing and mature leaves (data not shown). In the transverse section of a *N. benthamiana* leaf vein (Fig. 4.1C4 and Fig. 4.2A), TLCV was associated with phloem parenchyma, sieve elements, companion cells, vascular cambium and extended to the bundle sheath cells, but was not found in other cell types. Phloem, xylem and sieve elements are shown in a longitudinal section taken from leaf vein of TLCV-infected *N. benthamiana* (Fig. 4.2D). The identity of these cell types was determined by staining the sections with 0.1% toluidine blue (data not shown) (Mauseth, 1988). A similar tissue tropism was found in lateral root (Fig. 4.2G), stem (Fig. 4.2H) and leaf (Fig. 4.2I) tissue. In the stem section (Fig. 4.2H) the virus was associated with both inner and outer phloem cells and some adjacent cells in the cortex and pith. Vascular specificity of TLCV was also observed in *S. lycopersicum* and *N. tabacum*, although chromogenic signals were not as strong as in *N. benthamiana* (Fig. 4.2E and 4.2F). Unlike TLCV, TGMV was detectable in both vascular and non-vascular cells (Fig. 4.1C5 and Fig. 4.2J-4.2L), as reported earlier (Morra & Petty, 2000; Nagar et al., 1995; Wege et al., 2001).

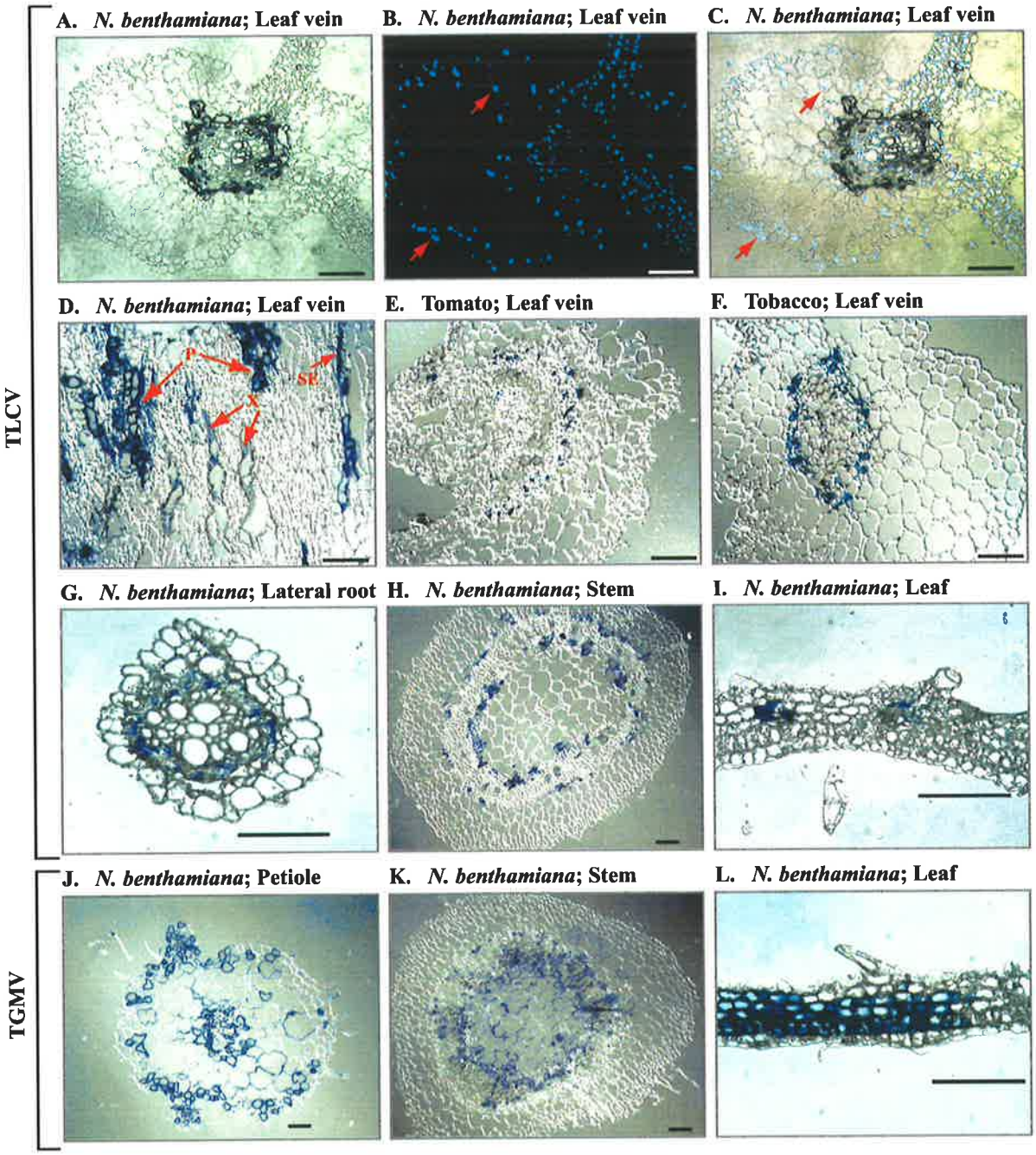


Figure 4.2. Comparative in situ localization of TLCV and TGMV. Tissue sections of TLCV-infected *N. benthamiana,* tomato and tobacco hybridized with TLCV-specific CS-cp DIG probe are compared with TGMV-infected *N. benthamiana* probed with TGMV CS-cp DIG probe. Sections were viewed with differential interference contrast (DIC) optics (D-L) or with a bright-field illuminator (A). Nuclei (red arrows) were identified by staining with DAPI (B). (C) is a merged image of (A) and (B). Tissue type, host plant and viral infection are indicated. All sections were transverse, except D which was longitudinal. P= phloem, X= xylem, SE= sieve elements. Bars = 100 μm.

4.3.3 TLCV nucleic acid accumulates in the cytoplasm

The majority of phloem cells produced strong hybridisation signals. These signals indicated the presence of TLCV nucleic acid in both cytoplasm and nucleus (Fig. 4.1C4 and Fig. 4.2A). Identity of the cytoplasm in fixed cells was confirmed by probing the healthy sections with a ubiquitin probe to detect ubiquitin transcripts (Fig. 4.3C). Nuclei were visualised by DAPI (4', 6-diamidino-2-phenylindole dihydrochloride) treatment, and were detected in the majority of cells (Fig. 4.2B). Absence of signals from some cells was presumably because nuclei occupy a small portion of the cell volume and therefore may have not been represented in the respective section. DAPI staining was masked by the strong chromogenic signals produced by the DIG-labelled probe (Fig. 4.2C). Merging of images obtained with the TLCV-specific probe and DAPI staining confirmed that viral nucleic acid was not confined to the nucleus only but was also present in the cytoplasm (Fig. 4.2C).

Strong chromogenic signals in the cytoplasm of TLCV infected cells were obtained with the CS-cp probe (Fig. 4.1C4 and Fig. 4.2A-I). This probe could detect both viral DNA and virion-sense transcripts (Table 4.2). To distinguish viral DNA from transcripts, RNA was removed enzymatically. Thus two serial sections from TLCV-infected tissue were prepared and one was treated with DNase-free RNase A in a NaCl-deficient buffer to digest both ss- and dsRNA (Mathews, 1991; Rezaian et al., 1991). These sections were hybridized with the homologous TLCV-specific CS-cp probe. RNase treated, TLCV-infected sections still exhibited chromogenicity in the cytoplasm (Fig. 4.3A and 4.3D). Ubiquitin mRNA in healthy sections was used as a control to verify the effectiveness of RNase treatment and to ensure that residual RNase was not interfering with hybridization of the riboprobes. The ubiquitin signal detected in healthy

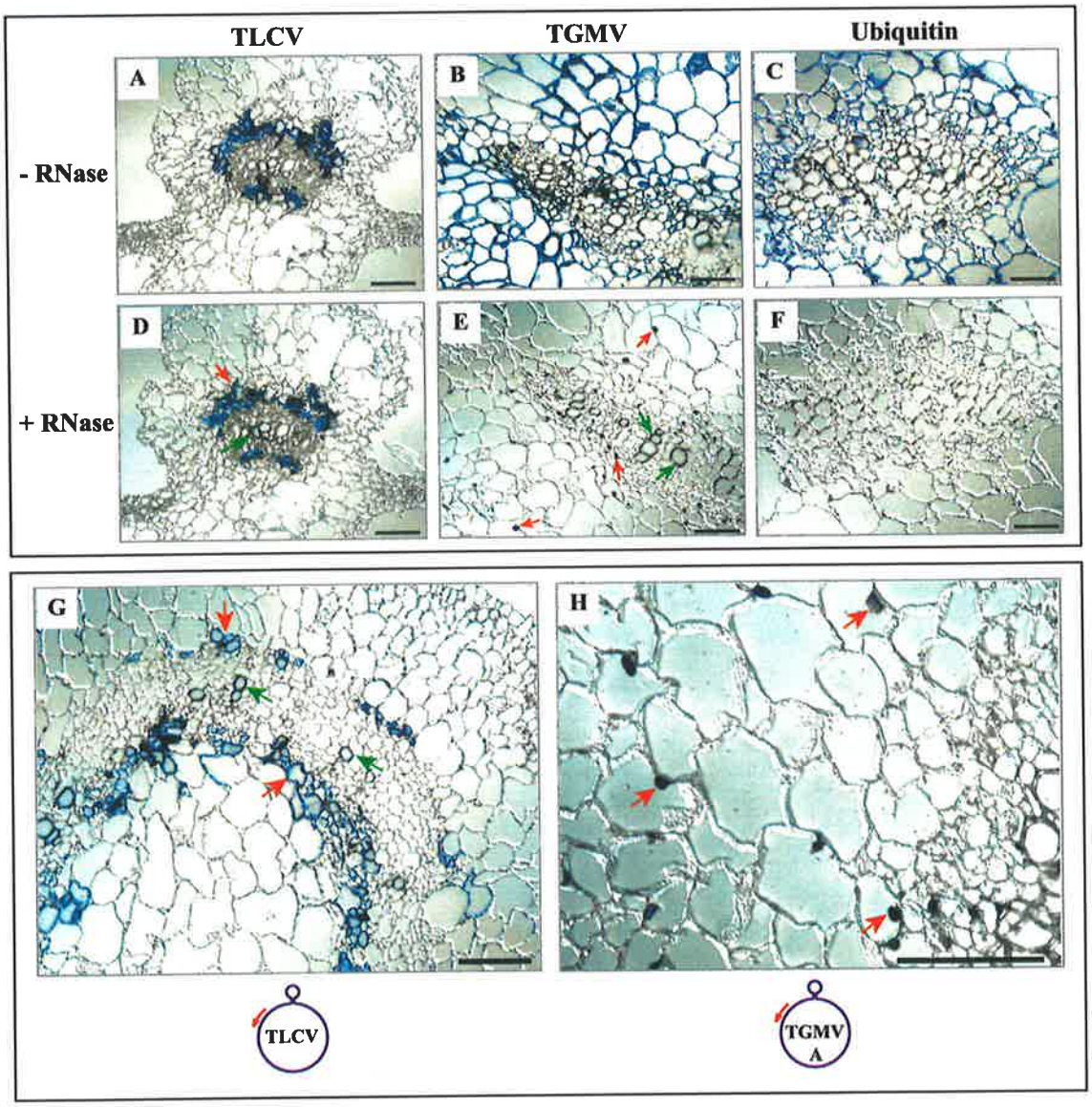


Figure 4.3. Differentiating viral DNA from transcripts. Transverse sections of leaf vein (A-F) and stem (G- H) of *N. benthamiana* showing hybridization of a TLCV-specific CS-cp probe with TLCV-infected sections (A, D) and a TGMV-specific CS-cp probe with TGMV-infected sections (B and E). The control was complementary-sense probe for ubiquitin gene transcripts hybridized with non-infected sections (C and F). –RNase, no enzyme treatment; +RNase, treatment with DNase-free RNase. Transverse sections of either TLCV-infected (G) or TGMV-infected (H) tissues were hybridized with CS-rep DIG-labelled probes. Red arrows indicate the nuclei containing TGMV DNA (E and H) and TLCV ssDNA (D and G) in phloem cells, and green arrows (D, E and G) indicate infected xylem. Bars = 100 μm.

sections (Fig. 4.3C) was lost after RNase treatment (Fig. 4.3F). Similarly, signals were retained in the nuclei of cells in sections derived from TGMV-infected plants after RNase treatment (Fig. 4.3E). These results are consistent with previous findings (Morra & Petty, 2000; Qin & Petty, 2001; Wege et al., 2001) that TGMV viral DNA is localized in nuclei (Fig. 3E) while the transcripts are present in the cytoplasm (Fig. 3B).

The identity of the cytoplasmic TLCV nucleic acid was also investigated by strand-specific probing. TLCV-infected sections probed with CS-rep, designed to detect viral DNA but not transcripts (Fig. 4.1A; Table 4.2), exhibited chromogenic signals in both the nuclei and cytoplasm of infected cells (Fig. 4.3G). This observation confirmed the results of the RNase treatment (Fig. 4.3A and 4.3D). As a control, a TGMV-specific CS-rep probe was hybridized to TGMV-infected tissues. Strong signals were observed only in the nuclei of TGMV-infected cells (Fig. 4.3H), consistent with results following the RNase treatment (Fig. 4.3E).

The study of subcellular DNA accumulation was extended to another monopartite species, *Tomato yellow leaf curl Sardinia virus* (TYLCSV). The number of infected cells in sections obtained from TYLCSV-infected *N. benthamiana* plants was considerably less than in sections from TLCV-infected plants, and chromogenic signals in TYLCSV-infected plants were always weaker than in TLCV-infected plants (compare Fig. 4.1C4, 4.2A and 4.3A with Fig. 4.4A). Using a strand-specific probe to detect TYLCSV DNA but not its transcripts (CS-rep; Table 4.2) chromogenic signals were mainly observed in the nuclei of infected phloem cells. Cytoplasmic signals were also present but these were relatively weak and limited to a few cells (Fig. 4.4B). Thus, the localization pattern of TYLCSV DNA in *N. benthamiana* appears to be intermediate

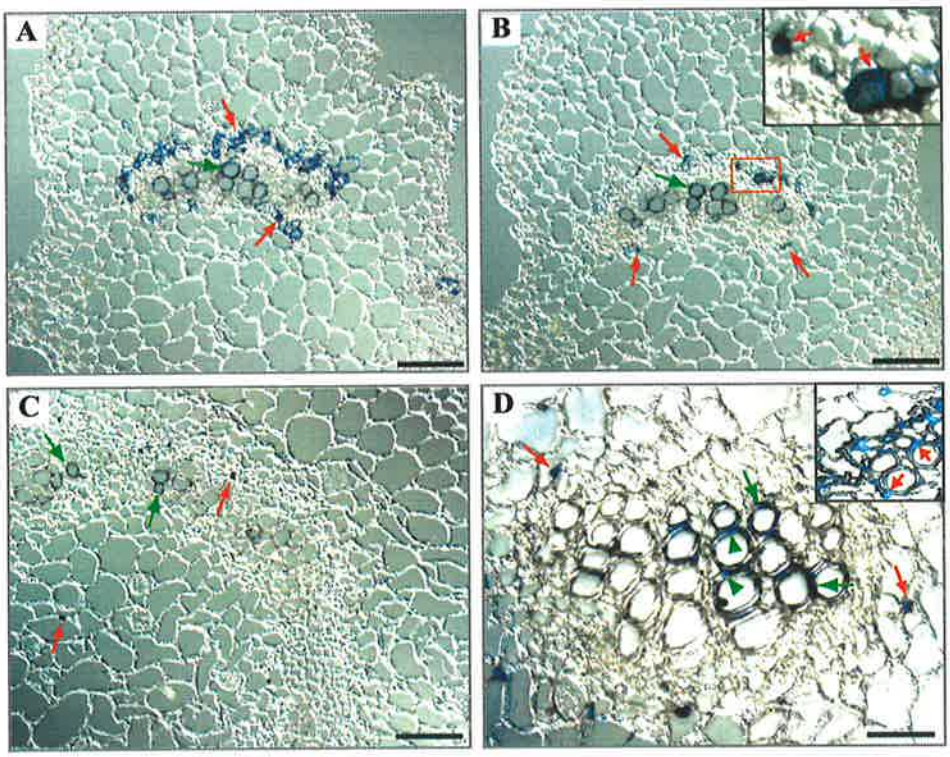


Figure 4.4. Localization of TYLCSV and TGMV in xylem. Localization of TYLCSV **(A and B)**. Transverse sections of leaf vein from TYLCSV-infected *N. benthamiana* hybridized with TYLCSV CS-cp (A) or CS-rep (B) probes. The area inside the red box is enlarged in the inset in B. Localization of TGMV in xylem **(C and D)**. Transverse sections of stem (C) and leaf vein (D) from TGMV-infected *N. benthamiana* hybridized with TGMV CS-rep probe. Red arrows show the viral- specific hybridization signals in phloem cells and green arrows indicate the viral-specific signals in xylem. Arrow heads in D show the spots of stronger hybridization signals indicative of xylem pits. Inset in D is the image of vascular cells stained with DAPI showing nuclei of immature xylem (red arrows) located next to developed xylem. Bars = 100 μm.

between TLCV and TGMV. It is possible that the low level of TYLCSV DNA signals reflects the reduced titre of this virus in *N. benthamiana* plants compared to TLCV. However, the pattern of TYLCSV DNA accumulation suggests that cytoplasmic localization is not unique to TLCV.

4.3.4 Cytoplasmic TLCV signals are essentially due to ssDNA

To determine whether cytoplasmic TLCV DNA was ss or ds, tissue sections were hybridized with TLCV-specific virion- and complementary-sense probes homologous to a part of the intergenic region (IR) (Fig 4.1A) which is not transcribed to a detectable level (Dry et al., 1993). CS-ir probe, designed to detect ss- and dsDNA forms (Fig. 4.1B and Table 4.2), produced strong chromogenic signals in the cytoplasm (Fig. 4.5B). The VS-ir probe, designed to target dsDNA only (Fig. 4.1B and Table 4.2), produced strong signals only in the nuclei (Fig. 4.5A) after a denaturation step (see chapter 3, section 3.2.12). This observation confirmed that dsDNA only contributed to signals from the nucleus, and suggested that cytoplasmic signals were due to ssDNA.

To further test this hypothesis, the target DNA in tissue sections was denatured by heat denaturation prior to hybridization (see chapter 3, section 3.2.12) with either CS-cp (Fig. 4.5F) or CS-rep probes (data not shown) and its chromogenicity compared with non-denatured sections. No difference in signal intensity was found between sections hybridized following denaturing (Fig. 4.5F) and non-denaturing treatments (Fig. 4.5C), indicating that TLCV ssDNA, not dsDNA, accumulates in the cytoplasm. Analysis of the DNA forms present in TLCV-infected plants by two-dimensional agarose gel electrophoresis has shown that ssDNA is the predominant form of viral DNA (Alberter et al., 2005). A higher level of ssDNA compared to dsDNA was also observed (Fig.

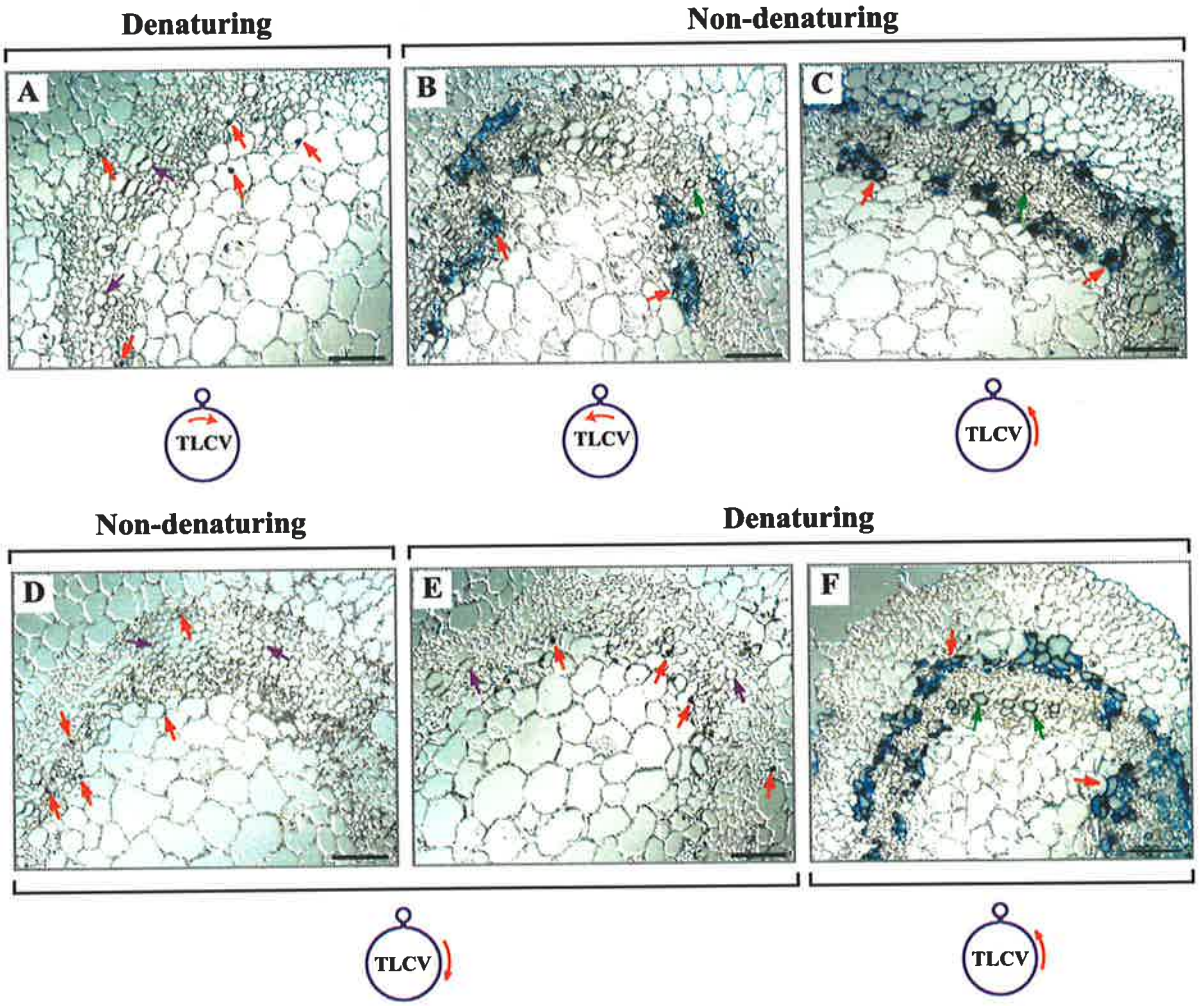


Figure 4.5. Differentiating ssDNA from dsDNA. Transverse sections of stem from TLCV-infected *N. benthamiana* hybridized with the probes indicated after denaturing and non-denaturing treatments as described in the methods. Red arrows indicate the nuclei containing the complementary-sense ssDNA (D) and the replicative form of TLCV dsDNA (A and E), red arrows (B, C and F) indicate TLCV ssDNA in phloem cells, green arrows (B, C and F) indicate infected xylem and purple arrows (A, D and E) indicate xylem vessels lacking viral signals. Bars = 100 μm.

4.1B). Nevertheless, signal intensity was stronger in denatured sections (Fig. 4.5E) than non-denatured ones (Fig. 4.5D) using a virion-sense probe that can detect viral dsDNA (VS-cp: Table 4.2), indicating that a proportion of nuclear signal represents dsDNA form. Faint staining signals, as seen in Fig. 4.5D, are probably due to the presence of cs ssDNA (see also Fig. 4.1B, lanes 1 and 5) known to be associated with geminivirus infection (Saunders et al., 1991). Together, these results indicate that TLCV ssDNA accumulates both in the cytoplasm and the nucleus while dsDNA is present in the nucleus only.

4.3.5 ssDNA of TLCV, TYLCSV and TGMV accumulates in xylem

Xylem vessels are inert tubes which are involved in water transport and provide mechanical support to plant tissues (Boyce et al., 2004). It is generally assumed that plant viruses move systemically through phloem, although some RNA viruses, including sobemoviruses (Moreno et al., 2004; Opalka et al., 1998; Schneider & Worley, 1959), a potyvirus (Dicenta et al., 2003) and a furovirus (Verchot et al., 2001) have been found in xylem. As yet, no DNA virus has been reported to localize to xylem vessels. Using probes specifically designed to detect ssDNA (Table 4.2; Fig. 4.1A), the ssDNA of three geminiviruses {TLCV (Fig. 4.2A, 4.2D, 4.3D,4.3G, 4.5B, 4.5C and 4.5F; see also Fig. 4.7), TGMV (Fig. 4.2J, 4.2K, 4.3E, 4.4C and 4.4D) and TYLCSV (Fig. 4.4A and 4.4B)} was observed in xylem vessels. It is important to note that no TLCV (Fig. 4.5A, 4.5D and 4.5E), TGMV or TYLCSV (data not shown) dsDNA was observed in the xylem.

Viral DNA was detected more frequently in the xylem of younger leaf veins than in stem sections (Table 4.3) taken immediately beneath the younger leaf (compare Fig.

Table 4.3. Localization of TLCV, TGMV and TYLCSV in xylem vessels

		TLCV	TYLCSV	TGMV
[a]**Leaf**	**H**	43 (59.5%)	45 (61.5%)	56 (59%)
	I	30 (40.5%)	34 (38.5%)	41 (41%)
[a]**Stem**	**H**	336 (87%)	332 (90.5%)	321 (88%)
	I	51 (13%)	35 (9.5%)	44 (12%)

[a] Five sections each derived from leaf midrib or stem were observed and target free (healthy; H) or target containing (infected; I) xylem vessels were counted

4.3D with 4.3G and Fig. 4.4D with 4.4C). Viral DNA was not detected in the xylem vessels of mature leaves (data not shown). Unlike other cell types where TGMV ssDNA accumulates in the nucleus (Fig. 4.4D; red arrows), xylem vessels exhibited TGMV ssDNA signals in the entire vessel (Fig. 4.4D; green arrows). This is because a mature xylem vessel does not contain nuclear and cellular contents (Fukuda, 1997). Small spots of high chromogenic intensity were also evident in some xylem vessels (green arrowheads; Fig. 4.4D), which probably represent the pits of xylem (see Fig. 4.7e) where more viral DNA may be accumulating.

Hybridization signals in xylem were shown to be virus specific by the negative results obtained with mock inoculated plants (Fig. 4.1C1) and with sections taken from virus infected plants incubated either without probes (data not shown) or with probes not specific to the inoculated virus (Fig. 4.1C2 and 4.1C3). Xylem localization data were further supported by the lack of hybridization signals in xylem vessels using probes designed to exclusively detect viral dsDNA (Fig. 4.5A and 4.5E) or ubiquitin (Fig. 4.3C and 4.3F).

4.3.6 Vascular restriction of TLCV is released by TGMV

The tissue tropism of a geminivirus can change in a mixed infection with another geminivirus. For example, the phloem-limited BGMV was shown to infect mesophyll cells in a mixed infection with TGMV (Morra & Petty, 2000). In TLCV and TGMV co-infected plants TLCV was mainly associated with vascular cells but could also infect a small number of mesophyll cells (Fig. 4.6B). To detect both viruses in the same section, CS-rep probes of TLCV and TGMV labelled with DIG and fluorescein respectively were applied simultaneously and detected sequentially (chapter 3, section 3.2.11).

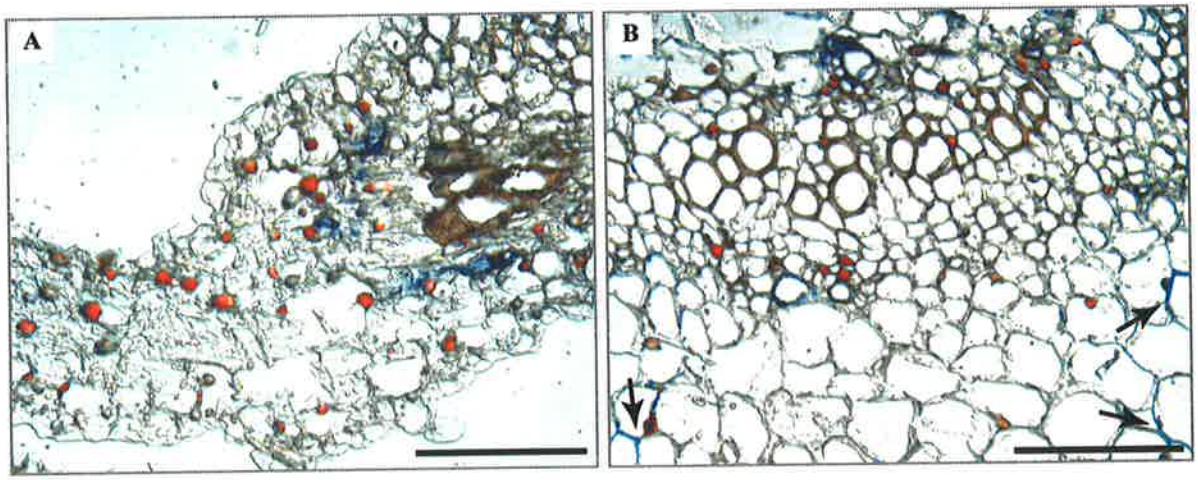


Figure 4.6. Detection of TLCV in mesophyll tissues co-infected with TGMV. Transverse sections from leaf lamina **(A)** and stem **(B)** taken from *N. benthamiana* plants co-infected with TLCV and TGMV were hybridized simultaneously with TLCV CS-rep DIG-labelled probe (blue) and TGMV Cs-rep fluorescein-labelled probe (red) and stained sequentially. Non-vascular cells infected by TLCV are indicated by arrows (B). Bars = 100 μm.

TGMV (red fluorescence) was found in the nuclei of vascular and mesophyll cells while TLCV (blue precipitate) was restricted mainly to vascular cells and was present in the cytoplasm and nuclei of infected cells (Fig. 4.6A and 4.6B). Examination of 12 tissue sections derived from stems, petioles and leaf veins of TLCV and TGMV co-infected plants identified 3212 TGMV-infected cells but only 855 TLCV-infected cells, of which 726 (~85%) were vascular-associated and the remaining 128 (~15%) were in the mesophyll. The majority of TLCV-infected cells (95%) also contained TGMV. However, a few TLCV-infected cells did not show TGMV DNA. It is not clear whether these cells did not contain TGMV DNA, or the red signals of TGMV DNA were completely masked by the strong blue signals produced by TLCV probe. Alternatively, the lack of TGMV DNA in those cells could be due to the absence of nuclei in the sections. It was also noted that the number of cells in which TGMV was detected depended on the probe used. A TGMV-specific probe, which could detect both viral DNA and transcripts, was able to identify many infected cells (Fig. 4.1C5 and Fig. 4.2J-L), whereas a TGMV-specific probe that could only hybridize to viral DNA detected a small number of infected cells (Fig. 4.6A and 4.6B). As explained earlier, this is probably the result of nuclei not being present in the cell sections due to their relatively smaller size. These results indicate that TGMV can partially alleviate phloem restriction of TLCV.

4.4 Discussion

Cell-to-cell movement of geminiviruses involves replication in nuclei, nuclear export of viral progeny and movement of virus through the cytoplasm to plasmodesmata (Gafni & Bernard, 2002). Previously, geminiviral DNA has been detected in the nuclei of infected cells (Morilla et al., 2004; Morra & Petty, 2000; Qin et al., 1998; Qin & Petty,

2001;Wege & Jeske, 1998; Wege et al., 2001). Consequently, DNA movement through the cytoplasm has been considered to be transient (Zhang et al., 2001). Here it is reported that TLCV ssDNA accumulates in the cytoplasm at significant levels, while replicative dsDNA is present in the nucleus. However, consistent with earlier findings, the cytoplasmic viral DNA was not detected in TGMV infections. The results suggest that these viruses may utilize different modes of DNA transport across the cytoplasm.

Three independent experiments, RNase digestion, strand-specific probing and hybridization under denaturing and non-denaturing conditions, confirmed that TLCV ssDNA accumulates in the cytoplasm. Morilla et al. (2004) recently reported that TYLCSV DNA localized predominantly in the nucleus. Results reported here are consistent with this in that the ssDNA of the phloem-specific monopartite TYLCSV was found to accumulate mainly in the nucleus, although some viral ssDNA was observed in the cytoplasm. It is possible that this discrepancy simply reflects the different in situ hybridization methods used in these studies. In this thesis, strand specific probing was used to differentiate viral DNA forms. Moreover, a proteinase K treatment was carried out in all in situ hybridisation experiments. Such a treatment may allow detection of viral ssDNA complexed with CP (Palanichelvam et al., 1998) which acts as a ssDNA binding protein in the cytoplasm. Supporting this idea is the observation that the CP of TYLCV is detectable in the cytoplasm of infected cells using immunolocalization, while a CP:GFP fusion protein expressed in the absence of other viral proteins localizes to the nucleus (Rojas et al., 2001). The cytoplasmic TLCV ssDNA may be encapsidated or present as a nucleoprotein complex with CP or with host ssDNA binding proteins. The significance of TLCV ssDNA accumulation in the cytoplasm and its possible role in viral movement is presently unknown.

It remains unclear whether ssDNA or dsDNA is involved in viral trafficking. Studies with bipartite begomoviruses are contradictory, supporting the involvement of ssDNA (Lazarowitz & Beachy, 1999; Padidam et al., 1999; Pascal et al., 1994), dsDNA (Rojas et al., 1998) or both ss- and dsDNA (Hehnle et al., 2004). Based on the finding that ssDNA of TLCV accumulates in the cytoplasm, and that ssDNA of the three viruses studied here is present in the xylem vessels (discussed below), it is suggested that ssDNA may be involved in the movement of these viruses.

It has been suggested that viral, host and environmental factors (Ding et al., 1999b; Kong et al., 2000; Morra & Petty, 2000; Wang et al., 1996) determine the tissue tropism of geminiviruses. The results described in this thesis indicate that TLCV is restricted to the vascular and bundle sheath cells of tissues derived from leaf, stem and root of different host plants at different developmental stages and inoculation period. This suggests that vascular limitation of TLCV is mainly determined by viral factors, and is consistent with the theory that phloem-limited viruses may lack cell-to-cell movement functions in non-vascular tissues (Taliansky & Barker, 1999). When co-infected with the mesophyll-invasive TGMV, it was found that the vascular-limitation of TLCV was partially abrogated. The mechanism by which TGMV can release phloem restriction of TLCV remains unclear.

In this study, geminiviral ssDNA was found in xylem tissue. A model depicting the route of viral entry to xylem cells is shown in Figure 4.7. Xylem arises from the vascular cambium through transdifferentiation (Fukuda, 1997). Immature xylem cells contain active nuclei (Mauseth, 1988). Such immature cells are evident in inset to Fig. 4.4D. Subsequently, these cells undergo programmed cell death and lose their nuclei

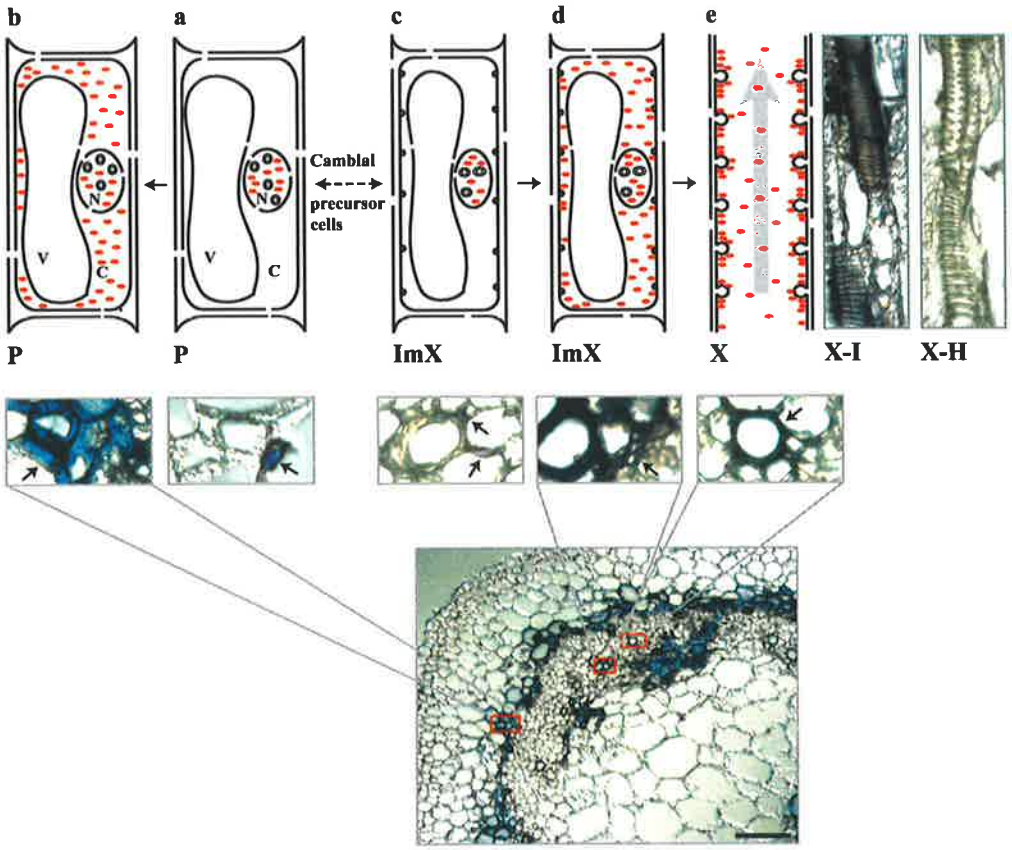


Figure 4.7. A model derived from the in situ localization studies to explain the distribution of TLCV ss- and dsDNA in the stem vasculature of *N. benthamiana*. (a) A replicative form of viral DNA (dsDNA) is present in the nucleus of phloem cells (P). Once a significant amount of ssDNA is synthesized in the nucleus it accumulates in the cytoplasm (b). During differentiation (c-e) of immature xylem cells (ImX) to xylem (X) through programmed cell death, which results in the loss of cellular contents, viral ssDNA either in the form of nucleoprotein complex or virion particles accumulates in the mature xylem vessels especially around the pits of xylem (e). Longitudinal sections of infected xylem (X-I) and healthy xylem (X-H) are shown. Lower panels shows the viral forms explained in the model hybridized with the probes specific to detect only dsDNA or both ss- and dsDNA as explained in the text.

and cell contents, leaving hollow dead cells that form vessels or tracheids (Fukuda, 1997). To explain the localization in mature xylem, it is proposed that geminiviruses preferentially localise to vascular cambium, comprising the most actively dividing cells in the vasculature (Mauseth, 1988), and then spread to phloem and xylem parenchyma by two separate processes: actively, by cell-to-cell movement, and passively, through the differentiation of cambium cells. Several lines of evidence support this hypothesis. First, TLCV DNA was not observed in mature leaves where cambium cells are not present (Mauseth, 1988). Second, a larger proportion of xylem vessels were infected in young leaves, where transdifferentation from cambium cells is highly active, than in stem tissue, where this process is largely absent. Third, the localisation of geminiviruses to xylem cells is supported by an increasing body of evidence implicating xylem tissue in long-distance transport of plant viruses (Dicenta et al., 2003; Moreno et al., 2004; Opalka et al., 1998; Schneider & Worley, 1959; Verchot et al., 2001). Opalka et al. (1998) proposed a model to explain the systemic movement of virus from apoplast to symplast whereby *Rice yellow mottle virus* (RYMV) chelates calcium in the pit membrane of xylem to destabilize the membrane structure for its movement to living immature tracheids. It must be noted that localization experiments in this study simply indicate the presence of geminiviruses in some of the xylem vessels and do not provide any information about the role of xylem in geminiviral transport.

It was possible that the viral DNA signal observed in xylem tissue was due to the agroinoculation procedure, where *Agrobacterium* containing viral DNA could be directly introduced to xylem cells. It has been observed that *Agrobacterium* cells were present in transformed tobacco plants, both into *in vitro* as well as *ex vitro* plants after transformation (Matzk et al., 1996). However, *Agrobacterium* has only been observed in

the samples taken from root zone of *ex vitro* plants and *Agrobacterium* has not been found in the aerial parts of the plant (Matzk et al., 1996). Moreover, there are no known reports indicating that *Agrobacterium* moves through xylem. Furthermore, the observed localization of viral DNA preferentially in young leaves compared to old leaves, which are closer to the site of inoculation, indicates that this is highly unlikely.

The finding that TLCV ssDNA accumulates in the cytoplasm may represent a new feature of the lifecycle of some geminiviruses. Moreover, the differential DNA accumulation pattern of TLCV and TGMV is suggestive of disparate movement mechanisms of monopartite and bipartite geminiviruses.

Chapter 5 – Analysis of *Tomato leaf curl virus* encoded proteins involved in viral movement

5.1 Introduction

Plant viruses encode one or more proteins for their cell-to-cell and long distance movement (Carrington et al., 1996). These proteins interact with the endomembrane system and plasmodesmata (Pd) to mediate cell-to-cell movement (Lazarowitz, 1999; Lucas & Gilbertson, 1994). Long distance movement involves the transport of virus via phloem tissues (Gilbertson & Lucas, 1996). Some plant viruses are unable to spread systemically however they are capable of replicating and spreading within inoculated leaves (Gilbertson & Lucas, 1996). This suggests that cell-to-cell and long distance movement may have distinct mechanism(s).

Having a DNA genome, geminiviruses have evolved mechanism(s) to transport DNA within the cell, between cells and between tissues and organs. In bipartite begomoviruses, DNA component A encodes the coat protein (CP), which is not essential for cell-to-cell and long distance movement (Padidam et al., 1995; Unseld et al., 2004). The DNA-B encodes two proteins, BV1 (also designated BR1) and BC1 (also designated BL1), that are required for viral movement. BV1 acts as a nuclear shuttle protein (NSP), exporting both ss- and ds- viral DNA from the nucleus (Noueiry et al., 1994; Sanderfoot & Lazarowitz, 1995). The BC1 protein, designated the viral MP, increases the plasmodesmatal size exclusion limit (SEL) and facilitates cell-to-cell transport of viral DNA (Noueiry et al., 1994). A specific interaction between NSP and MP is required for cell-to-cell movement (Sanderfoot & Lazarowitz, 1995). CP of

bipartite begomoviruses has been shown to complement NSP-deficient viruses (Ingham et al., 1995; Qin et al., 1998) and also assists the MP in cell-to-cell movement functions by its ability to affect the accumulation of viral ssDNA (Qin et al., 1998), indicating that CP has an indirect role in the movement of bipartite begomoviruses.

To date, the understanding of monopartite geminivirus movement is mostly based on studies of their bipartite counterparts. In contrast to the bipartite begomoviruses, the CP of monopartite begomoviruses is essential for systemic infection (Noris et al., 1998; Rigden et al., 1993) and appears to act analogously to the NSP of bipartite begomoviruses (Rojas et al., 2001). Two other proteins from monopartite begomovirus, V1 and C4, have been implicated in cell-to-cell movement and may have a functional similarity to the MP of bipartite geminiviruses (Rigden et al., 1993; Rojas et al., 2001). A derivative of TLCV containing a mutation in *v1* (TLCV-*V1* mutant) accumulated lower levels of ssDNA (Rigden et al., 1993). This result could reflect an ability of V1 to stabilize ssDNA (Padidam et al., 1999) or alternatively indicate a role of V1 in viral movement. Furthermore, the V1 protein from TYLCV demonstrated a limited capacity to move cell-to-cell, to interact with plasmodesmata (Pd) and increase their size exclusion limit, and to mediate cell-to-cell movement of DNA (Rojas et al., 2001). In addition, two studies have implicated TLCV *V1* in symptom expression. First, plants infected with an infectious construct of TLCV-*v1* were asymptomatic (Rigden et al., 1993). Second, transient expression of *V1* by a TMV based viral vector caused severe stunting of *N. benthamiana* and *N. clevelandii* plants (Selth et al., 2004). Similarly TLCV *C4* has produced severe phenotypic symptoms when stably expressed in transgenic plants (Krake et al., 1998). Moreover, plants infected with TLCV-*c4* (TLCV-*C4* mutant) have produced mild symptoms (Rigden et al., 1994). The observations that

movement proteins are determinants of pathogenicity (Duan et al., 1997; Hou et al., 2000; Ingham et al., 1995; Pascal et al., 1993) are consistent with the hypothesis that V1 and/or C4 play a role in cell-to-cell movement of TLCV.

In this study, an analysis of subcellular localization of the proteins potentially involved in the movement of TLCV is presented. Plasmolysis and brefeldin A (BFA) treatments were used to analyse the association of these proteins with endoplasmic reticulum (ER) and Golgi apparatus. In addition, a possible role of a host factor from tomato, SlUPTG1, which interacts with V1 protein of TLCV in viral movement, is discussed.

5.2 Materials and Methods

5.2.1 Plant material

Tobacco (*Nicotiana tabacum* cv. Samsun), transgenic tobacco carrying a *Cauliflower mosaic virus* (CaMV) 35S promoter-driven *C4* ORF cassette (Krake et al., 1998) and tomato (*S. lycopersicon* L. *var.* Money Maker) were used in this study. All plants were grown under glasshouse conditions at 25-30°C.

5.2.2 GFP expression vectors

A variant of the shuttle vector pART7 (Gleave, 1992), containing the full-length green fluorescent protein (GFP) ORF either upstream (with a start codon but lacking a stop codon; pART7-C'gfp) or downstream (without a start codon but containing a stop codon; pART7-N'gfp) of the multiple cloning site (T. Franks, unpublished data), was used to transiently express GFP fusion proteins in onion tissue. The *V1* ORF was amplified using primers P1 and P2 (Table 2.2), digested with EcoRI, and ligated into EcoRI/SmaI digested pART7-C'gfp to yield pART7-V1:GFP. The *V2* ORF was

amplified using primers P3 and P4 (Table 2.2), digested with EcoRI/BamHI, and ligated into similarly digested pART7-C'gfp to yield pART7-V2:GFP. *C4* was amplified using primers P5 and P6 (Table 2.2), digested with C4 EcoRI/BamHI, and ligated into similarly digested pART7-N'gfp to yield pART7-C4:GFP. The *V1* and *V2* ORFs were fused separately to the 3'end of GFP and the *C4* ORF was fused to the 5'end of GFP downstream of the CaMV 35S promoter. The *SlUPTG1* ORF (lacking a stop codon) was amplified with primers P33 and P34 (Table 2.2), digested with *Eco*RI and *Bam*HI, and ligated into similarly digested pN'gfp to generate an in-frame N-terminal fusion to the *GFP* gene. Control constructs were pART7-GFP, which expresses free GFP (T. Franks, unpublished data), and pBI121-H2B:YFP, which expresses *Arabidopsis* HISTONE 2B (H2B) fused to the GFP yellow variant yellow fluorescent protein (YFP) (Boisnard-Lorig et al., 2001).

The open reading frames encoding GFP:V1, GFP:V2, GFP:C4 and GFP were amplified by PCR using respective plasmids of pART7 mentioned above as DNA template and primer pairs P19/P21, P19/P22, P20/P23 and P19/P20 (Table 2.2) respectively. The PCR product was digested with XhoI and SpeI and ligated into the XhoI and SpeI sites of the plasmid pTA7002 (Aoyama & Chua, 1997). All clones obtained were verified by sequence analysis.

5.2.3 In situ hybridizations

Templates for the generation of RNA probes were amplified by the polymerase chain reaction (PCR) using TLCV-specific primers (Table 2.2). RNA probes labelled with fluorescein-12-UTP and digoxigenin-11-dUTP were prepared using Fluorescein or DIG

RNA Labelling Mix respectively (Roche Diagnostics, Castle Hill, Australia) as described in section 3.2.5.

Plant material was collected from TLCV-infected plants three weeks post-inoculation. Preparation of tissue sections, hybridization and detection of DIG- and fluorescein-labelled probes was carried out as described in section 3.2.

5.2.4 Transient expression of GFP fusion proteins using microprojectile bombardment and plasmolysis

Onion epidermal strips placed on agar containing Murashige and Skoog Salt Mixture (Invitrogen, Carlsbad, California) were bombarded with each of the vectors pART7 and pTA7002 GFP expression constructs (section 5.2.1) as described (section 2.2.24). After bombardment, tissue was stored in the dark for 24 h and GFP/YFP expression visualized using a Bio-Rad Radiance 2100 Confocal Laser Scanning Microscope (CLSM) System. The excitation wavelength of 488nm was used for both GFP and YFP analysis. For pTA7002 constructs, tissue was sprayed with a solution containing 30 μM dexamethasone (DEX) and 0.01% Tween 20 after 24 h of bombardment and visualized by CLSM after 24 h of DEX induction. After capturing the images, plasmolysis was performed by incubating the sections of onion epidermal layer with 0.5 M mannitol for 20 min and images recorded.

A sterilized Buchner funnel and vacuum flask was set up in the laminar flow to set the BY-2 cells on the sterile filter paper (Whatman 1, 4.25cm diameter). Four ml of 3 days old BY-2 cell culture was placed onto the filter paper and a gentle vacuum was applied to remove excess liquid. The filter paper carrying the BY-2 cells was transferred onto

solid MS_0 medium. The cells were bombarded as described (section 2.2.24) except with a pressure of 450 kPa. The transformed BY-2 cells were collected in an eppendorf tube containing MS_0 media using a needle under the stereo microscope. The collected cells were visualized using CLSM.

5.2.5 Plant infiltration

For agroinfiltration experiments, the V1:GFP, V2:GFP, C4:GFP, SlUPTG:GFP and ATG:GFP sequences downstream of the CaMV 35 promoter were released from pART7 plasmids by NotI digestion and ligated into NotI-digested pART27. *A. tumefaciens* strain C58 was transformed separately with each of these binary vectors by electroporation. *N. tabacum* leaf tissue was infiltrated with *A. tumefaciens* cells as described (Selth et al., 2004) prior to visualisation by confocal microscopy. Likewise pTA7002 GFP expression plasmids (section 5.2.1) were introduced into *A. tumefaciens* strain C58 for plant infiltration studies. The plants were sprayed with a solution containing 30 μM dexamethasone (DEX) and 0.01% Tween 20 after 24 h of infiltration. Samples were collected after 24 h of DEX induction and processed for CLSM.

5.2.6 Brefeldin A treatment and staining

An inducible system, pTA7002 GFP expression constructs (section 5.2.1), was used in BFA treatment experiments. Inducible system provides an opportunity to induce the expression of GFP fusion proteins after BFA treatment. After 20 h of agroinfiltration with pTA7002 GFP constructs, the abaxial side of the tobacco leaves were infiltrated with an aqueous solution of 50 μg ml^{-1} of BFA in 0.1% DMSO. After 3 h of BFA treatment, the plants were sprayed with a solution containing 30 μM dexamethasone (DEX) and 0.01% Tween 20 and after 6 h of induction samples were collected for

CLSM. In a control experiment, samples were collected after 6 h of induction without BFA treatment.

Callose was stained by incubating the onion epidermal layer and tobacco leaf samples in 0.01% aniline blue fluorochrome (Biosupplies, Parkville, Australia) in 10 mM Na phosphate buffer pH 9.2 for 40 min in the dark. ER staining was done by incubating the BY-2 cells in an aqueous solution containing 100 μg/ml of rhodamine B-hexyl ester (Invitrogen) for 5 minutes.

5.3 Results

5.3.1 Mutations in TLCV *V1* and *C4* ORFs partially affect viral movement

Unlike most of the bipartite begomoviruses, the coat protein (CP) is essential for systemic infection by monopartite begomoviruses (Noris et al., 1998; Rigden et al., 1993). Earlier, we reported that disruptions in V1 and C4 ORFs of TLCV resulted in symptomless or attenuated systemic infections, respectively, in tomato plants (Rigden et al., 1993; Rigden et al., 1994). However, mutations in the V1, V2 and C4 ORFs did not disrupt viral replication (Rigden et al., 1993; Rigden et al., 1994). The *V1* and *C4* genes of another monopartite begomovirus, TYLCV, have been shown to be involved in cell-to-cell movement of virus (Rojas et al., 2001).

To analyze the role of the *V1* and *C4* genes in TLCV movement, we inoculated tobacco plants with wild type (wt) TLCV and derivatives having mutations in the *V1* and *C4* genes and analysed viral DNA accumulation by in situ hybridization. Fewer infected cells were observed in sections inoculated with both the *c4* (Fig. 5.1E) and *v1* (Fig. 5.1G) mutants compared to sections inoculated with wt virus (Fig. 5.1C). Moreover,

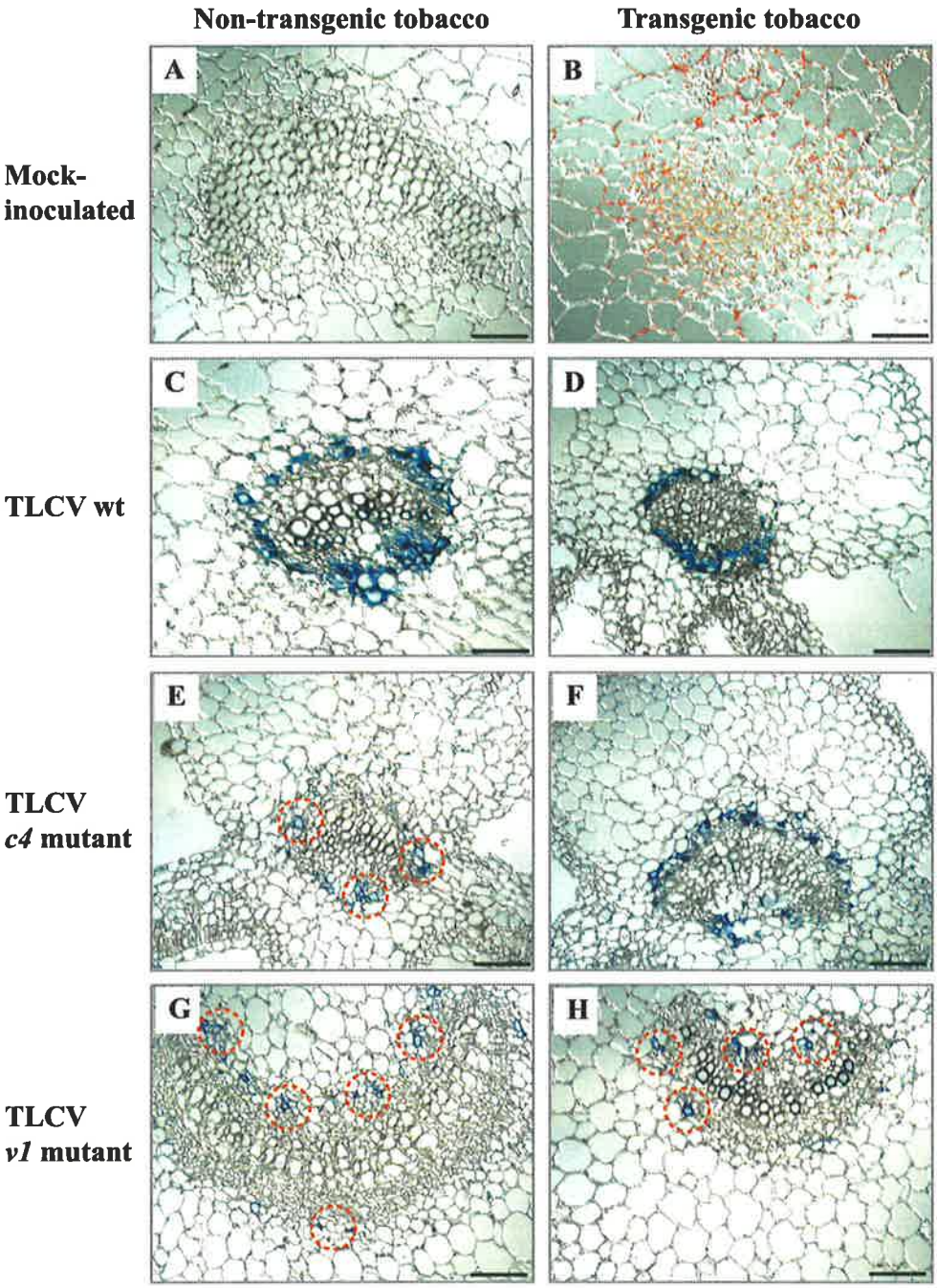


Figure 5.1. Localization of TLCV wild type (wt), *v1* and *c4* mutants in *C4* expressing transgenic and non-transgenic tobacco. Transverse sections of leaf vein were hybridized with TLCV-specific probes. The probes were digoxigenin-labelled, complementary-sense ssRNA from *V2* ORF (A and C-H) and fluorescein-labelled, virion-sense ssRNA from the *C4* ORF (B). Dashed circles in E, G and H show the groups of infected phloem cells. Bars = 100 μm.

infected cells were observed in the patches of 1-3 infected cells per patch as indicated by red dashed circles (Fig. 5.1E, 5.1G and 5.1H). This suggests that the cell-to-cell movement of TLCV *v1* and *c4* mutants is less efficient than the wt virus. To confirm that the lack of C4 protein was responsible for the observed deficiencies in movement, we inoculated wt and mutant TLCV to transgenic tobacco plants expressing the *C4* gene from TLCV under the control of the 35S promoter (Krake et al., 1998). In these plants, the *c4* mutant infected as many cells as the wt virus (Fig. 5.1F and 5.1D), while movement of the *v1* mutant remained inefficient (Fig. 5.1H), suggesting that complementation of the *c4* mutant occurred solely by the *C4* transgene product. It is interesting to note that in transgenic plants where *C4* is expressed in all cell types (Fig. 5.1B), the wt and mutant viruses were unable to cross the vascular barrier (Fig. 5.1D, 5.1F, 5.1H).

Previously it has been shown that TLCV *v2* mutant is unable to cause systemic infection (Rigden et al., 1993). Here, in situ hybridization results have shown that a *v2* mutant was replicating in the infected cells (Fig. 5.2B) when delivered biolistically to detached leaf. Moreover, *v2* mutant virus was found in all cell types including epidermal, mesophyll and phloem cells suggesting that the virus was delivered to those cells by gold particle(s) (Fig 5.2B, red arrows). It is important to note that in the majority of the cells infected by *v2* mutant, the virus was confined to the single cell (indicated by red arrows) and was unable to infect the neighbouring cells (Fig. 5.2B) compared to TLCV wt (Fig. 5.2A). However, a group of 2-3 infected cells (indicated by green arrow) was observed. It is not clear whether the *v2* mutant was delivered to a single cell and then moved to the neighbouring cells or whether the mutant virus was delivered biolistically to each of the cells in that group. Together these results suggest that the *v2* mutant was

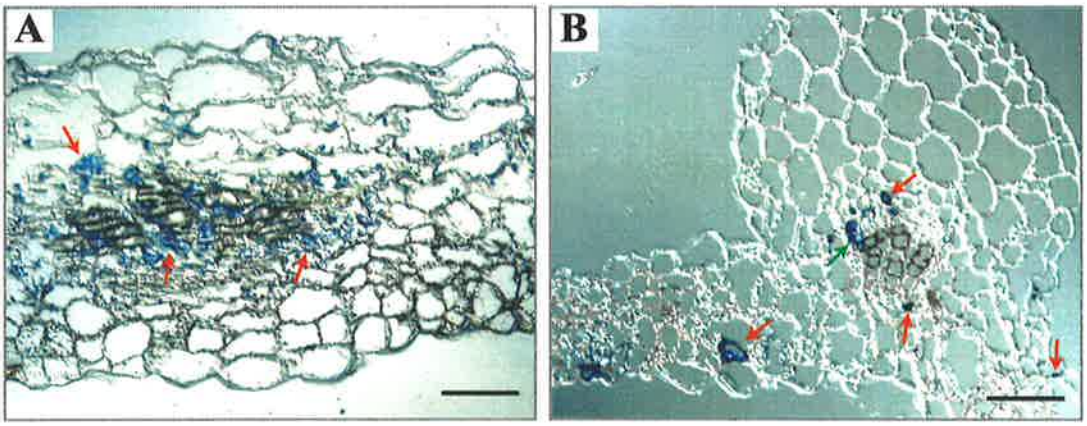


Figure 5.2. Localization of TLCV wild type **(A)** and *v2* mutant **(B)** in detached leaves of *N. benthamiana*. Transverse sections of leaf vein were hybridized with TLCV-specific digoxigenin-labelled, complementary-sense ssRNA from *V2* ORF. Red arrows indicate the hybridization signals of the virus. Green arrow shows a group of cells infected with virus. Bars = 100 μm.

replicating in the inoculated cells and was less efficient in infecting the neighbouring cells than the TLCV wt virus.

5.3.2 Subcellular localization of TLCV V1, V2, and C4 proteins

Proteins encoded by geminiviruses control cell-to-cell movement (Gafni & Bernard, 2002; Lazarowitz & Beachy, 1999). Three virus-encoded proteins have been implicated in the movement of the monopartite TYLCV (Rojas et al., 2001). The results of viral DNA accumulation of TLCV derivatives having mutations in *V1*, *V2* or *C4* ORFs have suggested that theses proteins have a role in viral movement (section 5.3.1). To further gain an insight into the role of V1, V2, and C4 proteins encoded by TLCV in virus movement, their subcellular localization was analysed in onion and tobacco epidermal cells. The fusion proteins (V1:GFP, V2:GFP and C4:GFP) were transiently expressed in onion cells following biolistic delivery, and in tobacco cells by agroinfiltration, and analyzed by confocal laser scanning microscopy.

Free GFP was distributed in both the cytoplasm, often in association with cytoplasmic strands, and the nucleus of bombarded cells (Fig. 5.3A). When optical sections taken through a cell expressing V1:GFP were combined, the pattern of fluorescence observed was similar to free GFP (Fig. 5.3B). However, analysis of single optical sections demonstrated that V1:GFP was primarily localised to the cell periphery (Fig. 5.3C and E), with ER (Fig 5.3D and 5.4A, white arrow) and around or probably inside the nucleus (Fig. 5.3D, white arrow). A similar peripheral but a distinct perinuclear pattern of fluorescence has been observed in *N. tabacum* protoplasts and *N. benthamiana* epidermal cells expressing TYLCV V1 fused to GFP (TY-V1:GFP; (Rojas et al., 2001)). In this study, the authors used an ER-specific stain to show colocalisation of

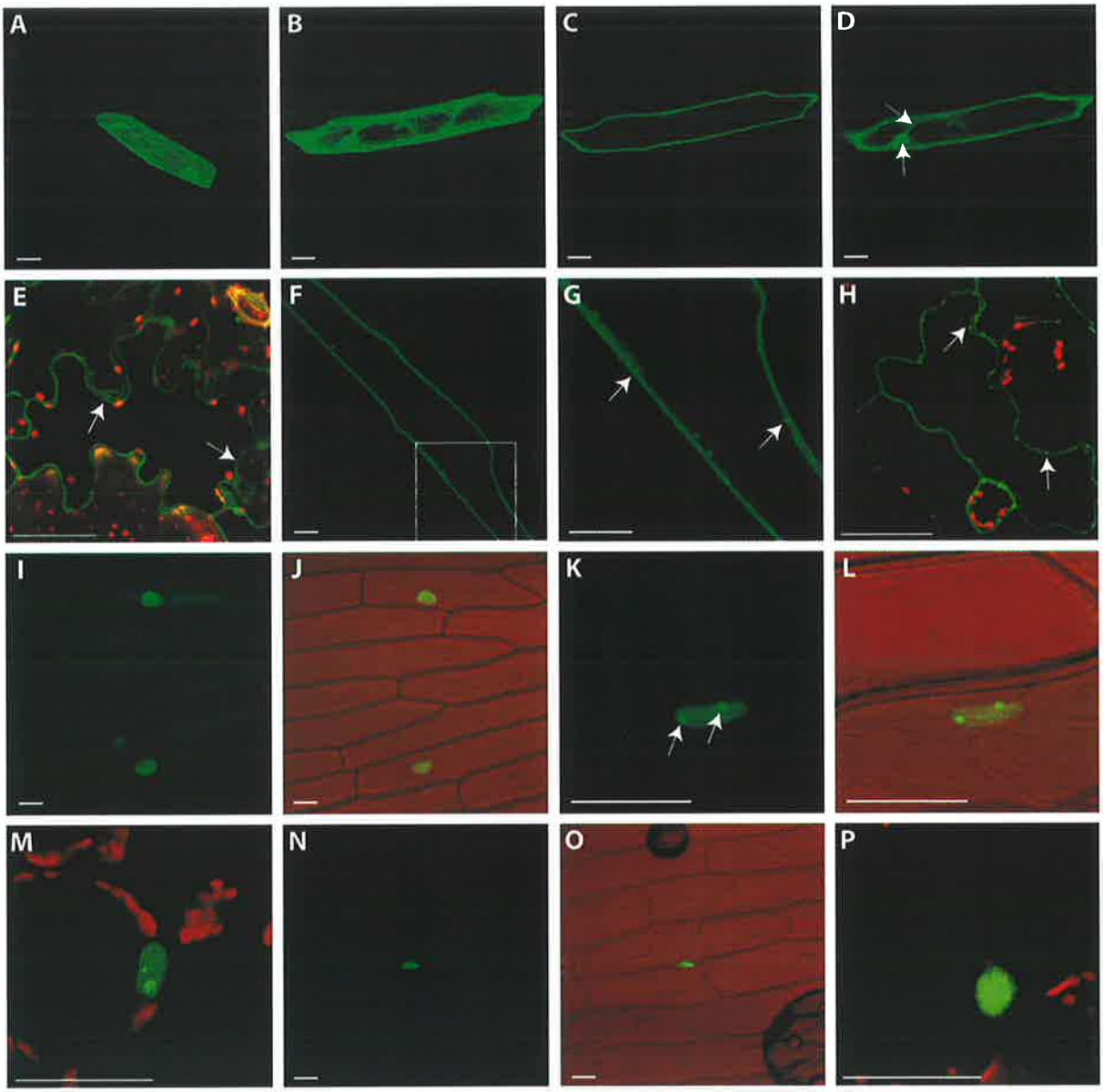


Figure 5.3. Subcellular localization of V1-, V2-, and C4-GFP fusion proteins. Confocal images of onion (A-D, F-G, I-L and N-O) or tobacco (E, H, M and P) epidermal cells. Combined optical sections from a cell expressing free *GFP* **(A)** or *V1:GFP* **(B)**. Single optical sections showing V1:GFP targeting the cell periphery **(C and E),** the nucleus, and associated with endoplasmic reticulum as indicated by white arrows **(D)**. C4:GFP showing localization to the cell periphery **(F-H)** and accumulating as small, discrete punctae (**G**; white arrows). V2:GFP showing localization to nuclei **(I-M)** and accumulation in nucleoli indicated by white arrows **(K and L)**. Nuclear localization of H2B:YFP **(N-P)**. Merged V2 **(J and L)** or YFP **(O)** with differential interference contrast (DIC) images showing the location of the nuclei in relation to the cell. Chloroplasts in tobacco are shown as red in E, H, M and P. Bars = 25 μm.

V1-GFP with the ER. The similarity between the fluorescence of V1:GFP in onion epidermal cells and TY- V1:GFP in *Nicotiana* cells and protoplasts suggests that TLCV V1 may also associate with the ER network. This is further confirmed by staining the BY-2 cells with an ER-specific stain and co-localization of V1:GFP with the ER (Fig. 5.5B). Likewise, an association of V1:GFP with cytoplasmic punctate bodies (Fig. 5.3E, white arrows, see also Fig. 5.7B), consistent with a localisation pattern seen in 20-30% of protoplasts transfected with TY-V1:GFP, suggest the association of V1:GFP with Pd.

In cells bombarded with C4:GFP, punctate fluorescence was consistently observed at the cell periphery (Fig. 5.3F and 5.3H) at either the plasma membrane or Pd (Fig.5.3G). This pattern of GFP expression has been observed for many MP:GFP fusions (Blackman et al., 1998; Padgett et al., 1996; Ryabov et al., 1998) and suggests that C4 is localized at Pd.

Nuclear localization of V1:GFP was also observed in onion and tobacco epidermal cells (Fig. 5.3D, 5.6I and 5.7B, white arrows). The nuclear localization pattern of V1:GFP was in contrast to the perinuclear localization of TY-V1:GFP and therefore it was further confirmed by determining its localization pattern in BY-2 cells and comparing it with the localization of C4:GFP. GFP fusion proteins were transiently expressed in BY-2 cells and images were taken at different planes to get a series of images. A nuclear localization of V1:GFP was observed (Fig. 5.4A, white arrow) compared to a perinuclear pattern of C4:GFP (Fig. 5.4D, white arrow). The nuclei were detected by DAPI staining (Fig. 5.4B and 5.4E) and nuclear and perinuclear localization was confirmed by merging images (Fig. 5.4C and 5.4F). V1:GFP signals were observed inside the nucleus in all the serial sections of the nuclear images (see Appendix-1,

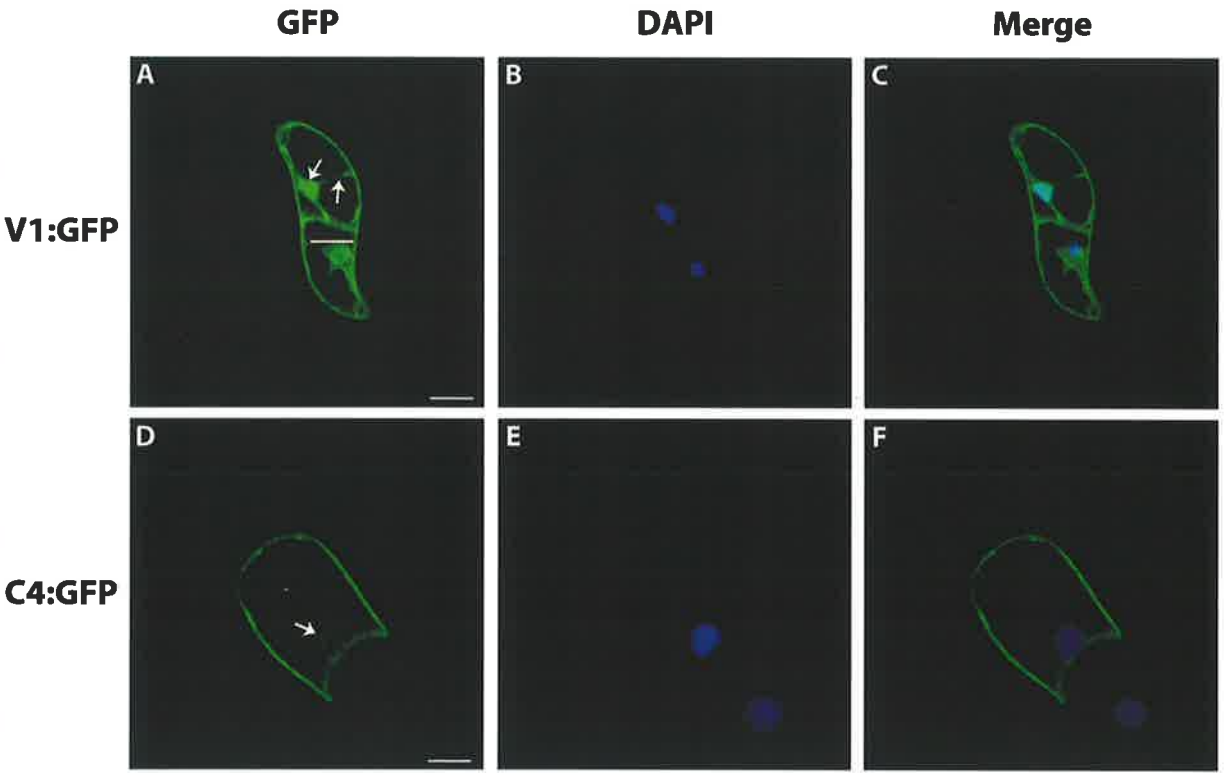


Figure 5.4. Localization of V1- and C4-GFP fusion proteins in BY-2 cells. Nuclear localization of V1:GFP indicated by arrow **(A)**. Perinuclear localization of C4:GFP indicated by arrow **(D)**. Nuclei were identified by DAPI **(B and F)**. A merged image of V1:GFP/DAPI **(C)** and C4:GFP/DAPI **(F)**. Localization of V1:GFP with endoplasmic reticulum (ER) is shown by arrow in A. Bars = 25 μm.

panels C-H). In another experiment, V1:GFP was co-delivered with an *Arabidopsis* HISTONE 2B::yellow fluorescent protein (H2B:YFP) which acted as a control for nuclear localization (Boisnard-Lorig et al., 2001). A nuclear localization of V1:GFP was evident when both V1:GFP and H2B:YFP transiently expressed in the same cell of *N. benthamiana* (Fig. 5.5A).

V2:GFP was found only in the nuclei of onion epidermal cells (Fig. 5.3I, 5.3J, 5.3K, 5.3L and 5.3M). In contrast to H2B:YFP fusion protein, which stains the whole nucleus evenly (Fig 5.3N, 5.3O and 5.3P), a significant amount of V2:GFP congregated in nucleoli (Fig. 5.3K and 5.3L). A similar pattern of nucleolar fluorescence was observed in *N. tabacum* protoplasts transfected with TYLCV CP:GFP and BDMV BV1:GFP fusion proteins (Rojas et al., 2001), indicating that subnuclear targeting of geminiviral coat and nuclear shuttle proteins is conserved and may play an important role in geminivirus infection.

5.3.3 Transiently expressed V1:GFP and C4:GFP associated with Pd

Microscopy of epidermal cells transiently expressing V1:GFP and C4:GFP demonstrated that these proteins localized to cell periphery (Fig 5.6E, 5.6F, 5.6I and 5.6J). In addition, punctate bodies were observed, which often formed paired foci (5.7B, arrowheads) suggest that there is an association of both V1 and C4 with Pd enriched cell wall regions. Similar types of paired foci were also observed in the cell wall when Pd associated RGPs were stably expressed in *N. benthamiana* plants (Sagi et al., 2005). To confirm this Pd association, onion epidermal cells were subjected to plasmolysis and the localization pattern was compared before and after plasmolysis. This treatment results in retraction of the plasma membrane (PM) from the cell wall, leaving Pd implanted in the

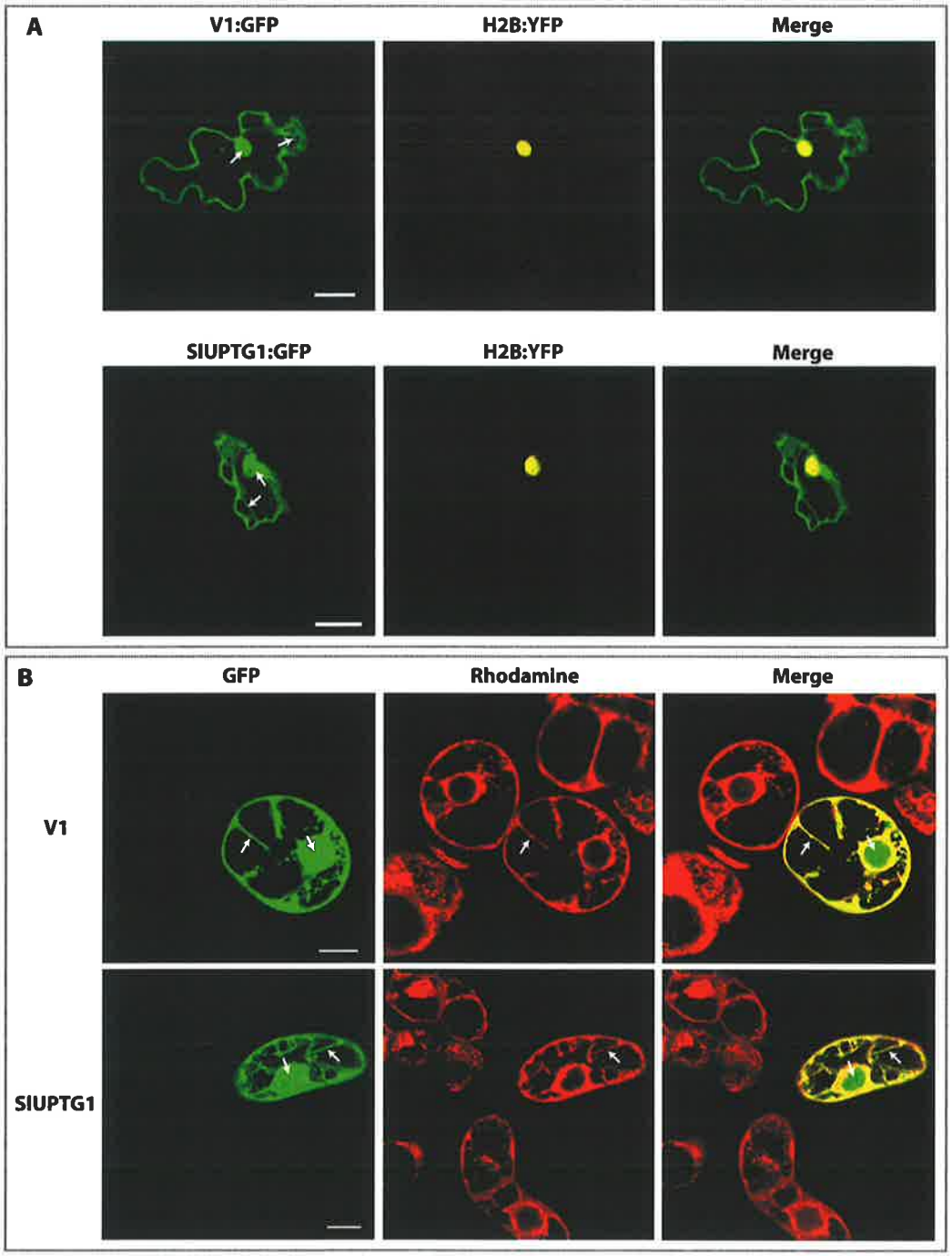


Figure 5.5. Subcellular localization of V1- and SlUPTG1-GFP fusion proteins. **(A)** Shows nuclear localization of V1 and SlUPTG1 when codelivered and expressed with H2B:YFP in the same cell of *N. benthamiana*. **(B)**. Shows association of V1 and SlUPTG1 with endoplasmic reticulum (ER) and nucleus in BY-2 cells. Red fluorescence represents ER stained by rhodamine B-hexyl ester. Signals associated with nucleus and ER are indicated by white arrows in A and B. Merged images are shown to indicate the sites of colocalization. Bars = 25 μm.

cell wall (Turner et al., 1994). Plasmolysis was induced in the onion epidermal cells 24 h after bombardment and then viewed under the CLSM. V1:GFP and C4:GFP fluorescence remained between plasma membrane (PM) and cell wall after plasmolysis (Fig. 5.6G, 5.6 H, 5.6K and 5.6L). No fluorescence was found between PM and cell wall of the plasmolysed cells which were expressing free GFP (Fig. 5.6C and 5.6D). Furthermore, fluorescent threads were observed in a few cells expressing V1:GFP and C4:GFP (Fig. 5.6G and 5.6K, white arrows). It has been reported (Hecht, 1912) that plasmolysis results in the formation of thin membranous structure known as Hechtian strands that anchor the PM to the cell wall. Recently, CLSM has been used to demonstrate that many Hechtian strands originate at or close to the entrance of Pd (Oparka et al., 1994) and these strands are associated with ER (Pont-Lezica et al., 1993). Lang-Pauluzzi (2000) has shown that Hechtian strands were formed to all sides of the protoplast in onion inner epidermal cells. Combined optical sections of V1:GFP after plasmolysis clearly showed that the fluorescent threads remained attached to the cell wall when the protoplast retracted (Fig. 5.6M).

To verify that the fluorescence spots inside cell walls indeed represent Pd, callose [(1→3)-β-glucans] was stained with aniline blue flurochrome. Callose is a component of specialized walls or wall associated structures, such as cell plates, plasmodesmatal channels, sieve plates and transient walls of microsporogenic and megasporogenic tissues (Stone & Clarke, 1992). Callose, therefore, has been widely used as a plasmodesmal marker (Baluska et al., 1999; Bayer et al., 2004; Gorshkova et al., 2003). Onion epidermal cells when treated with aniline blue displayed fluorescent labelled spots in the cell wall (Fig. 5.6N) indicative of Pd. Together these results suggest that

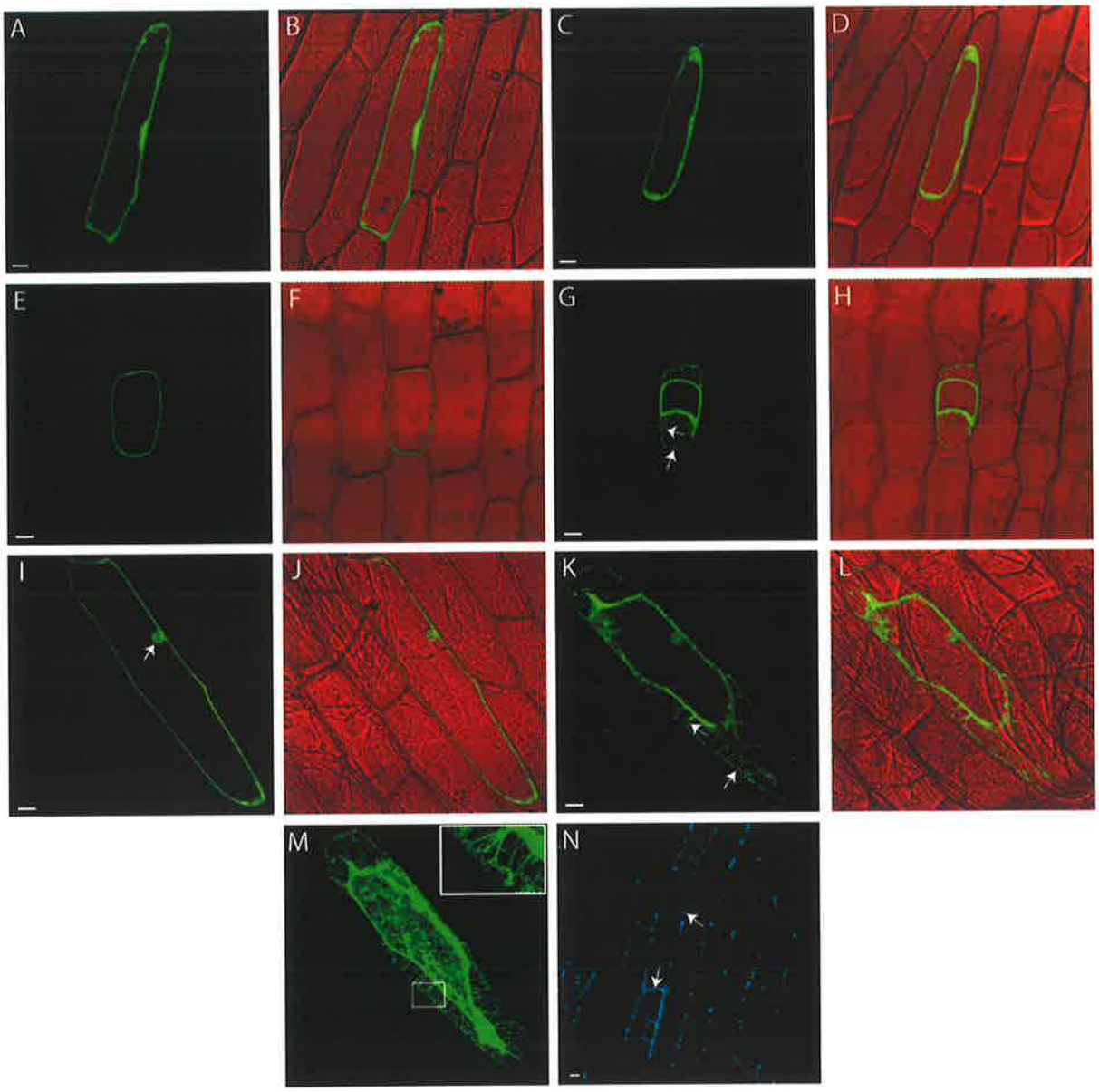


Figure 5.6. Subcellular localization of GFP, V1:GFP and C4:GFP in onion epidermal cells before (A, B, E, F, I and J) and after (C, D, G, H, K, L and M) plasmolysis. Single optical sections showing free GFP **(A-D)**, C4:GFP **(E-H)** and V1:GFP **(I-L)**. Hechtian strands and signals in cell wall are indicated by white arrows in G and K. Nuclear localization V1:GFP is indicated by an arrow in I. Merged images of GFP with differential interference contrast (DIC) are shown in B, D, F, H, J and L. Panel M shows the combined optical sections of V1:GFP. The area inside the white box is enlarged in the inset in panel M. Cell wall and plasmodesmata stained with analine blue flurochrome indicated by white arrows and are shown in N. Bars = 25 μm.

GFP:V1 and GFP:C4 fluorescent threads are related to Hechtian attachment sites and that these proteins have an association with Pd.

5.3.4 V1:GFP and C4:GFP transported to Pd via the Golgi apparatus

It has been suggested that the Golgi vesicles during cell division are involved in building primary Pd through the developing cell wall (Jones, 1976; Robards & Lucas, 1990). The possibility that V1:GFP and C4:GFP are transported to Pd via the Golgi apparatus was examined. BFA, a disrupter of the Golgi apparatus, was used to test this hypothesis. Epidermal cells of tobacco leaves treated with BFA during the transient expression of C4:GFP, V1:GFP and V2:GFP were compared with cells not treated with BFA. BFA treated cells displayed diffuse fluorescence with small punctate structures in the cell wall with V1:GFP and C4:GFP (Fig. 5.7B). Interestingly BFA treatment did not disrupt the localization of V2:GFP (Fig. 5.7B). These results suggest that targeting of V1:GFP and C4:GFP to cellular membrane and Pd probably occurred through the Golgi apparatus.

5.3.5 Localization of C4:GFP to cellular membrane is rapid and transient

Subcellular localization studies of C4:GFP indicated its cell peripheral localization, and in contrast to V1:GFP, C4:GFP was never observed inside the cytoplasm (Figs. 5.3, 5.4, 5.6 and 5.7). To understand how C4:GFP travelled to the PM after its synthesis in the cytoplasm, a time course experiment was conducted. *N. benthamiana* leaves were agroinfiltrated, sprayed with DEX and a section of the treated leaf was put under the CLSM and images were taken at 1 h time intervals. Faint signals of GFP fluorescence were observed in some cells after 1 h of induction (Fig. 5.8). The level of signal intensity gradually increased with time and a larger number of GFP expressing cells was

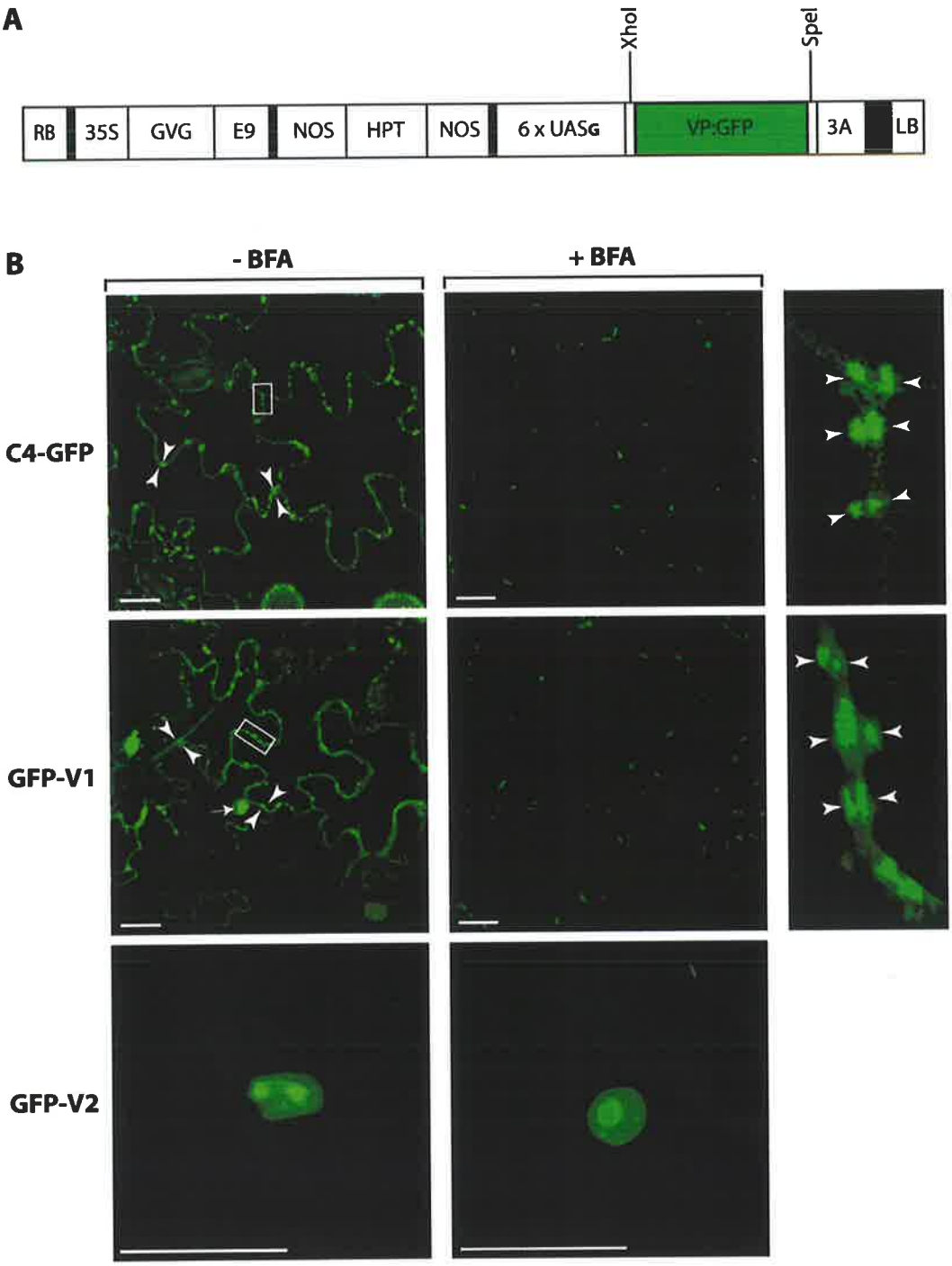


Figure 5.7. Localization of GFP fusion proteins using inducible system. **(A)** pTA7002 containing GFP fusion constructs used for induction experiments. VP:GFP denotes each of V1:GFP, V2:GFP and C4:GFP. **(B)** comparison of GFP:C4, GFP:V1 and GFP:V2 without (-BFA) and with (+BFA) brefeldin A treatment. Aroow heads in B indicate the paired foci in cell wall and arrow shows the nucleus. The area inside the white boxes is enlarged in the right of respective panel. Bars = 25 μm.

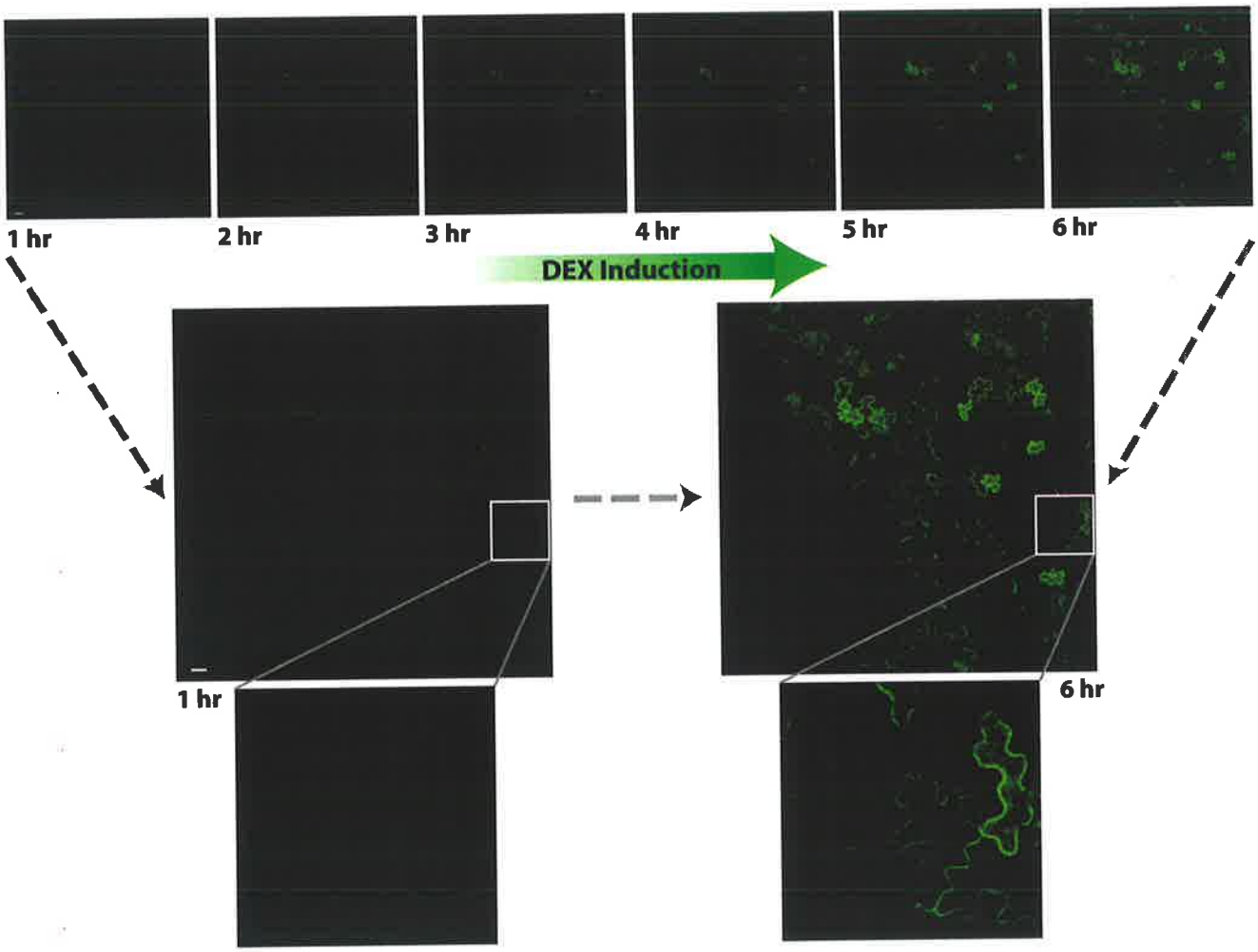


Figure 5.8. Time-scale analysis of C4:GFP localization. Images were captured after dexamethasone (DEX) induction. Time period is indicated. Area shown in white boxes is enlarged. Bar = 25 μm.

observed 6 h after induction compared with 5 h (Fig. 5.8). It is interesting to note that a significant intensity of GFP fluorescence was found in the PM suggesting that C4:GFP fusion protein is expressed in the cytoplasm and targeted to the PM immediately after its synthesis and accumulates in the PM and can be visualized when its accumulation reaches to a detectable level (Fig. 5.8).

5.3.6 Subcellular localization of V1:GFP and SlUPTG1:GFP fusion proteins

It has been shown in our laboratory that V1 interacts with a protein from tomato designated SlUPTG1, which is closely related to a family of reversibly glycosylated peptides (Selth et al., 2006). In addition, *SlUPTG1* expression significantly enhances the accumulation of TLCV ssDNA (Selth et al., 2006) suggesting that SlUPTG1 may have a role in viral infection. To further understand the role of SlUPTG1 in viral infection, subcellular localization of SlUPTG1 was examined using GFP fusion constructs expressed from the CaMV 35S promoter and its localization pattern was compared with V1. The subcelluar localization pattern of SlUPTG1 was similar to the localization of V1 in that it was present inside the nucleus (Fig. 5.5A) and was associated with ER (Fig. 5.5B). Single optical sections of onion cells expressing SlUPTG1:GFP showed fluorescence mainly at the cell periphery (Fig 5.9I and 5.9J), a pattern similar to that observed for V1:GFP (Fig. 5.9E and 5.9F). Fluorescence associated with expression of free GFP also appeared to localize at the cell periphery when single optical sections were analyzed (Fig. 5.9A and 5.9B). However, combined optical sections revealed that free GFP was also distributed throughout the cytoplasm and nucleus of onion cells (Fig. 5.3A).

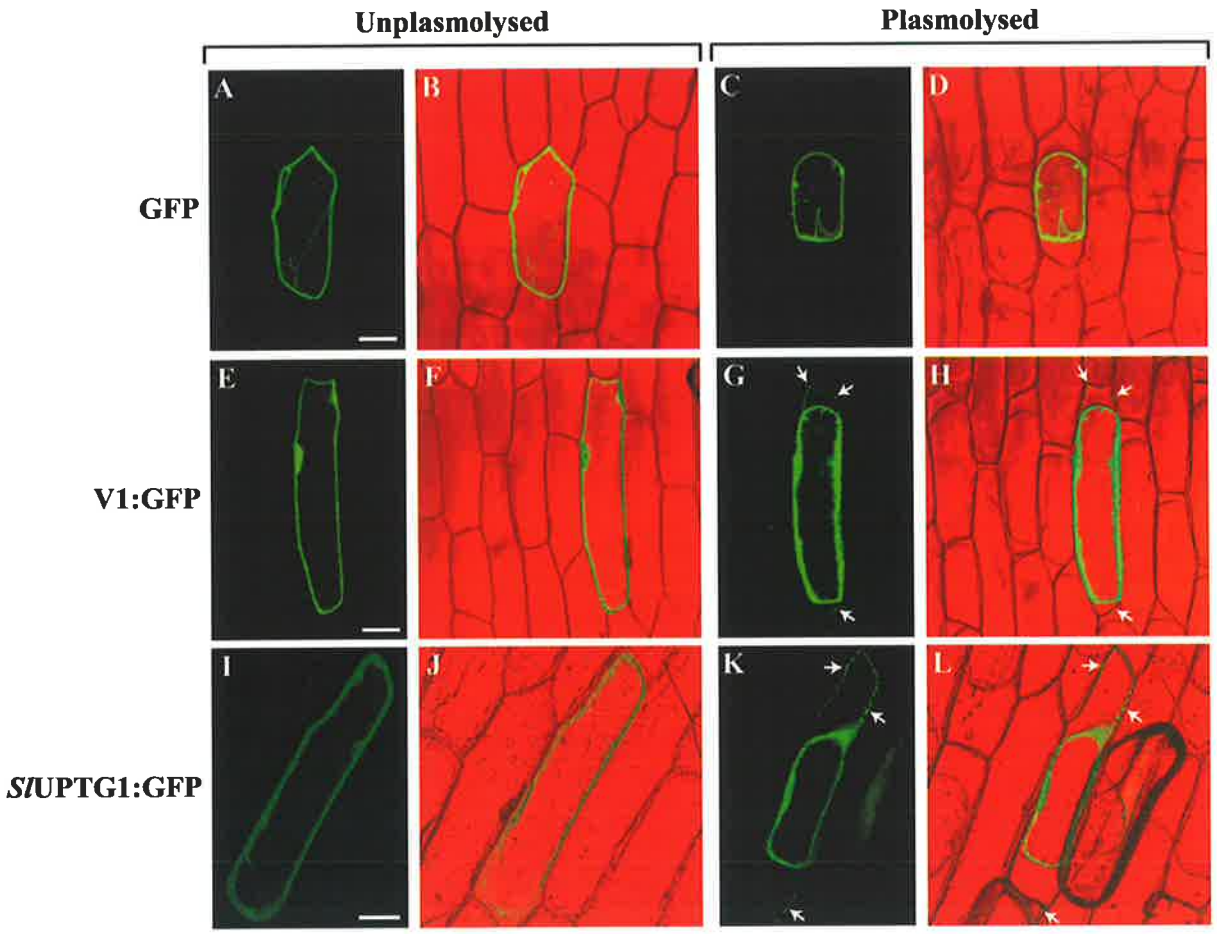


Figure 5.9. Subcellular localization of V1 and SlUPTG1. *V1:GFP* **(E-H),** *SlUPTG1:GFP* **(I-L)** and *GFP* alone **(A-D)** were transiently expressed in onion epidermal cells. Cells were analyzed for GFP fluorescence before and after plasmolysis. Punctate fluorescence at the cell wall of onion cells expressing either *V1:GFP* (G and H) or *SlUPTG1:GFP* (K and L) after plasmolysis is indicated by arrows. GFP images (A, C, E, G, I and K) and GFP/differential interference contrast (DIC) merge images (B, D, F, H, J and L) are shown. Bars = 50μm.

The four class 1 reversibly glycosylated polypeptides (^{C1}RGPs) from *Arabidopsis*, AtRGP1-AtRGP4, and a ^{C1}RGP from maize, SE-WAP41, are shuttled to Pd via the Golgi apparatus, leading to the suggestion that Pd association may be a general characteristic of ^{C1}RGPs (Sagi et al., 2005). To test whether SlUPTG1 and V1 also associate with cell wall components, cells expressing GFP-fusion proteins were subjected to plasmolysis. After plasmolysis, V1:GFP (Fig. 5.9G and 5.9H) and SlUPTG1:GFP (Fig. 5.9K and 5.9L) fusion proteins were observed mainly in the protoplast but also at punctae along the cell wall, a pattern indicative of Pd localisation. In contrast, free GFP was observed only within the protoplast and not in the zone between the PM and cell wall (Fig. 5.9C and 5.9D).

It must be noted that these transient experiments do not provide any information regarding the temporal and spatial localization of V1 and SlUPTG1 during TLCV infection. However, the results of this study suggest that SlUPTG1 would co-localize with V1 at the cell membrane and possibly at Pd if these proteins were produced in the same cells *in planta*.

5.4 Discussion

The monopartite begomoviruses have a single DNA molecule which contains all the genes required for viral replication, movement and pathogenicity. In this study, three genes encoded by the monopartite begomovirus TLCV were analysed for their role in viral movement. The results of this study are consistent with the report (Rojas et al., 2001) that three TLCV encoded proteins V1, V2 (CP) and C4 may contribute to viral movement. V1:GFP displayed a cell peripheral localization pattern and also showed fluorescent spots in the cell wall indicative of Pd association. Moreover, the V1:GFP

fusion protein produced a pattern of fluorescence reminiscent of ER localization. MPs from RNA viruses (Heinlein et al., 1998; Laporte et al., 2003) and DNA viruses (Ward et al., 1997) interact with ER and this is imperative for intra- and inter-cellular trafficking (Lazarowitz & Beachy, 1999). This suggests that V1 may have a role in trafficking of TLCV DNA. In contrast, the C4:GFP protein was found exclusively at the cell periphery and accumulated in small punctate bodies, a pattern indicative of Pd localization. Like the C4 protein from TYLCV (Rojas et al., 2001), TLCV C4 contains a putative N-terminal myristoylation site (GSLIS) from amino acids 4-8. N-terminal myristoylation domains are involved in membrane localization (Johnson et al., 1994) and this sequence may therefore play a role in the association of C4 with the PM. Pd localization has been reported for many plant viral MPs (Blackman et al., 1998; Rojas et al., 2001; Ryabov et al., 1998).

Pd are cytoplasmic connections between neighbouring plant cells and facilitate cell-to-cell cytoplasmic transport through cell walls (Robards & Lucas, 1990). Plant viruses modify Pd for cell-to-cell movement and then long distance spread of infection (Ding et al., 1999a). It is known that Golgi vesicles are involved in the formation of both primary and secondary Pd (Robards & Lucas, 1990). Sagi et al. (2005) have shown that ^{Cl}RGPs are Pd-associated proteins and are transported to Pd via the Golgi apparatus. The fungal toxin BFA has been widely used as an inhibitor of protein trafficking in the endomembrane system of a variety of eukaryotic cells (Fujiwara et al., 1988; Nebenfuhr et al., 2002). BFA has been shown to release the COPI coats (coat protein I vesicles) from the Golgi resulting in disruption of vesicle formation (Ritzenthaler et al., 2002). Association of V1 and C4 proteins with Pd and targeting of these proteins to Pd via

Golgi apparatus is supported by our observation that BFA treatment has significantly affected the V1:GFP and C4:GFP localization.

Together, these results suggest that the TLCV V1 and C4 proteins are involved in cell-to-cell movement of the viral DNA. Two lines of evidence from our in situ hybridisation studies support this idea. First, wild-type TLCV was able to infect significantly more cells than either its *v1* or *c4* mutant derivatives and cells infected by the mutant viruses tend to occur in clusters of phloem cells, suggesting that their cell-to-cell movement is inefficient. Second, the movement of the TLCV *c4* mutant virus was promoted by endogenous C4 protein supplied by a transgene. Disruption of any one of these ORFs did not completely stop the viral movement but resulted in reduced cell-to-cell movement. However, V2 is essentially required for systemic movement though *v2* mutant virus replicated in the inoculated cell. These results are also supported by the previous observation that V1 and C4 from TYLCV have a limited involvement in cell-to-cell movement (Rojas et al., 2001).

Given that the TLCV *v1* mutant accumulates lower levels of viral DNA (Rigden et al., 1993), and that V1 interacts with SlUPTG1 in yeast and *in vitro* (Selth et al., 2006) and SLUPTG1 enhances the accumulation of TLCV DNA (Selth et al., 2006). This suggests that SlUPTG1 could simply act to transport V1 to the plasma membrane, Pd and/or the Golgi apparatus where it would execute its putative movement-associated function. This idea is reinforced by our observation that both SlUPTG1 and V1:GFP may localize to the PM, ER and specifically to Pd. This is further supported by the observation that other ^{C1}RGPs from *Arabidopsis* and maize are associated with Pd (Sagi et al., 2005).

The question arises as to whether the presence of C4 protein, a putative movement factor of TLCV which may interact with Pd, would allow movement of TLCV out of the vasculature if the protein was expressed in non-vascular cells. In *C4* expressing transgenic plants, TLCV was unable to cross the vascular barrier. We can envisage two possible explanations for the inability of TLCV to enter mesophyll cells in these transgenic plants if indeed this protein has a role in movement. First, the C4 protein alone may be insufficient for allowing mesophyll invasion. Second, the *C4* transgene may be silenced upon TLCV infection while replication of the virus is not affected (Chapter 6) (Bian et al., 2006). This suggests that TLCV replicates in phloem cells and induces a gene silencing mechanism which spreads from vascular cells to non-vascular cells and causes silencing of the transgene, but this silencing is not functional in the phloem cells where the virus is replicating. This is conceivable because TLCV, like other geminiviruses, encodes a suppressor of RNA silencing (Selth et al., 2004). Such an explanation is in agreement with our observation that a TLCV-derivative containing a mutated *c4* ORF is complemented by C4 proteins in the transgenic plants.

The localization of TLCV CP to nuclei and nucleoli is also a characteristic of the NSP from bipartite begomoviruses. It is worth noting that CP accumulates preferentially in the nucleolus where ribosomal packaging occurs, suggesting that viral packaging or formation of nucleoprotein complexes may occur in this compartment. A similar nuclear localization for the CP of monopartite begomovirus TYLCV (Rojas et al., 2001) and monopartite mastrevirus, MSV (Liu et al., 2001) has been reported. Interestingly a nuclear localization of V1 was also found which was in contrast to the perinuclear localization of V1 from TYLCV (Rojas et al., 2001). However, perinuclear localization was found with C4 and such perinuclear localization was never observed with V1. The

discrepancy of this result remains unresolved. However, nuclear and perinuclear localization of V1 and C4 respectively and Pd association of both proteins explains the functional role of these proteins and raises the possibility that mutation in any one of these ORFs complements the function of the other. This possibility is supported by the observation that both *v1* and *c4* mutant viruses did not prevent viral infection and have a limited cell-to-cell movement.

Folding of newly synthesized proteins is a well-established fact (Jaenicke, 1991) and protein aggregation is an important event of cellular functions (Kopito 2000). Cells avoid accumulation of toxic aggregates by two mechanisms (1) suppression of aggregate formation by molecular chaperons and (2) the degradation of misfolded proteins by proteoasomes (Kopito 2000). The degrading of proteins disturbs their localization pattern. The localization results of this study suggest that V1-, V2- and C4-GFP fusion proteins were not degraded by the mechanisms suggested by Kopito (2000).

The results of this study demonstrate that TLCV CP performs functions similar to begomovirus BV1 which acts as a NSP. Neither TLCV V1 nor C4 proteins behaves like previously studied MP, including BC1 of the bipartite begomoviruses. Both proteins function cooperatively for efficient cell-to-cell movement.

Chapter 6 – Phloem specific TLCV induces systemic silencing and escapes from this silencing

6.1 Introduction

Eukaryotic cells suppress foreign genetic elements through a sequence-specific RNA silencing mechanism (Voinnet, 2001) known as gene silencing in plants (Napoli et al., 1990; van der Krol et al., 1990), RNA interference in animals and quelling in fungi (Cerutti, 2003; Pickford & Cogoni, 2003). RNA silencing can be triggered by RNA viruses (Burton et al., 2000; Jones et al., 1999), DNA viruses (Al-Kaff et al., 1998; Kjemtrup et al., 1998), antisense transcripts (Nellen & Lichtenstein, 1993), highly expressed-sense transcripts (van der Krol et al., 1990) and transgene loci producing double-stranded RNA (dsRNA) (Smith et al., 2000). A key feature of RNA silencing is the presence of small interfering RNA (siRNA), which acts as an intermediate in the silencing pathway (Hamilton & Baulcombe, 1999; Voinnet, 2001). Recent studies show that dsRNA is cleaved into siRNAs of about 21-25 nucleotides (nt) by RNase III-like enzymes named Dicer (Bernstein et al., 2001; Hamilton & Baulcombe, 1999; Zamore et al., 2000). siRNA is used as a guide for the degradation of single-stranded RNA (ssRNA) by an siRNA/ribonuclease complex known as the RNA-induced silencing complex (RISC) (Hammond et al., 2000).

RNA silencing in plants also involves RNA-directed DNA methylation (RdDM), in which DNA homologous to a triggering RNA is methylated *de novo* (Mette et al., 2000; Pelissier et al., 1999). RdDM occurs at both symmetric cytosine sites (CpG and CpNpG, where N is A, T, C or G) and at asymmetric sites (CpHpH, where H is A, T or C). Cao

et al. (2003) proposed that the initial establishment of RdDM requires the enzyme known as domains rearranged methylase (DRM), which is guided by siRNA to the target sequences. The maintenance of CpG methylation is accomplished by methyltransferase, whereas CpNpG and CpHpH methylation is maintained by chromomethylase and DRM redundantly (Cao et al., 2003).

siRNA homologous to the promoter region of a target gene induces transcriptional gene silencing (TGS), which is associated with promoter methylation. If siRNA is homologous to the coding region of the target gene, it induces posttranscriptional gene silencing (PTGS), which involves sequence-specific RNA degradation and methylation of the coding region. PTGS and TGS are mechanistically related as both involve the production of siRNA (Sijen et al., 2001).

RNA silencing in plants is considered to be an adaptive defence mechanism against viruses (Voinnet, 2001; Waterhouse et al., 2001). It is induced in response to viral replicating forms of RNA, presumably to degrade the invasive viral genomes. Plant endogenous or introduced genes that have homology with an infecting viral RNA could also be silenced (Jones et al., 1999; Jones et al., 2001). This process is known as virus-induced gene silencing (VIGS). It has been found that DNA viruses, including geminiviruses, are also capable of inducing gene silencing (Al-Kaff et al., 1998; Atkinson et al., 1998; Chellappan et al., 2004b; Kjemtrup et al., 1998).

The TLCV genome replicates in the nucleus of infected cells (see chapter 4) (Rasheed et al., 2006) by a combination of rolling circle replication (RCR) and recombination-dependent replication (RDR) (Alberter et al., 2005). Earlier it was found that TLCV

infection induces transcriptional silencing of TLCV promoter-driven β-glucuronidase (GUS) transgenes and causes methylation of the virus-derived promoter (Seemanpillai et al., 2003). Interestingly, the viral replication appeared not to be inhibited by the silencing (Seemanpillai et al., 2003). In line with this observation, it has been shown that expression of a truncated version of TYLCSV Rep protein (Rep-210) in transgenic tomato plants produces resistance to TYLCSV infection (Brunetti et al., 1997) but the resistance is overcome by transgene silencing (Lucioli et al., 2003). Furthermore, RNA silencing triggered by *C2* hairpin RNA in tomato was found to be ineffective against TLCV (Bian et al., 2006).

This chapter describes experiments to investigate transgene silencing by methylation. Transgenic tobacco plants carrying the TLCV *C4* ORF driven by the CaMV 35S promoter have been used to determine the level of transgene methylation and expression following TLCV infection. In addition the methylation levels of replicative form (RF) of TLCV DNA were determined. These results demonstrate that TLCV infection induces silencing of the virus-derived transgene, but that the virus escapes this defence mechanism, possibly due to a differential methylation of the *de novo* synthesized viral and host plant DNA.

6.2 Materials and methods

6.2.1 Plant materials.

Tobacco (*N. tabacum* cv. Samsun), tomato (*S. lycopersicum* L. *var.* Money Maker) and *N. benthamiana* were grown under glasshouse conditions at 25-30°C. Transgenic tobacco lines described previously (Krake et al., 1998), carrying a CaMV 35S promoter-driven *C4* ORF cassette, were grown under containment.

6.2.2 TLCV infectivity assays.

Plants at the 4-5 leaf stage were inoculated with an overnight culture of *A. tumefaciens* (strain C58) containing a TLCV dimeric construct in pBin19 (Dry et al., 1993). Developing leaves from individual plants (50 ~ 100 mg) were sampled and homogenized with 0.5 M NaOH at a ratio of 4µl per 1 mg tissue. The presence of TLCV DNA was tested by dot-blot analysis (Stonor et al., 2003).

6.2.3 Preparation of TLCV-specific RNA probes.

TLCV primers corresponding to the *V2* (P3 and P4, Table 2.2) and *C4* (P5 and P6, Table 2.2) ORFs were used to amplify DNA fragments by polymerase chain reaction (PCR). The DNA was ligated into a pGEM-T Easy vector (Promega, Madison, WI). The clones were linearized by suitable restriction enzymes and used as templates for transcription using T7 or SP6 DNA-dependent RNA polymerase (Promega) in the presence of α^{32}P-UTP according to the manufacturer's instructions. RNA probes used for in situ hybridization were labelled with fluorescein-12-dUTP (for the probe from complementary sense gene) and digoxigenin-11-dUTP (for the probe from virion sense gene) using Fluorescein or DIG RNA Labeling Mix respectively (Roche Diagnostics, Castle Hill, Australia).

6.2.4 Gel blot analysis.

Total RNA was extracted from expanding leaves (100 mg) using the TRIzol reagent (Invitrogen, Carlsbad, California) according to the manufacturer's instructions. mRNA was isolated from the total RNA by a poly-A mRNA extraction kit (Promega). mRNA obtained from 20 µg total RNA was analyzed in a 1.2% agarose MOPS gel containing 2 M formaldehyde (Selth et al., 2004). siRNA analysis of total RNA (5µg) was carried

out by electrophoresis in a denaturing 15% polyacrylamide-7 M urea gel using a Mini-PROTEAN 2 electrophoresis system (BioRad, Hercules, CA) (Wang et al., 2001). The nucleic acids were blotted onto Hybond$^+$ membrane (Amersham Biosciences, USA) and hybridized with ^{32}P-labeled, TLCV RNA probes at 42°C overnight in 50% formamide, 7% SDS, 250 mM NaCl and 125 mM NaH_2PO_4. The membrane was washed as described (Wang et al., 2001) and hybridization signals were detected using Kodak X-ray film. Southern blot analysis was carried out as described (Dry et al., 1993).

6.2.5 In situ hybridization.

Leaf samples were collected from TLCV-inoculated (at 60 days post inoculation (dpi)) and non-inoculated 35S:*C4* transgenic and non-transgenic plants. The tissues were fixed (see section 3.2.2) and sections, 6-8μm thick, were placed on silane-coated slides (ProSciTech, Thuringowa Central, Australia). Pre-hybridization, hybridization and post-hybridization were performed as described in chapter 3. Probes were detected using Fast Red or NBT/BCIP staining solutions (Table 3.1). The slides were dehydrated, mounted in entellan mounting medium and were observed under a light microscope (Axioscope2, Germany).

6.2.6 Bisulfite DNA sequencing.

Bisulfite modification and sequencing were performed as described (Grunau et al., 2000). The *5S* rRNA genes in the treated samples were sequenced as a control using the primers reported (Fulnecek & Matyasek, 1998) to monitor bisulfite conversion rate. It was assumed that the cytosines in symmetric (CpG and CpNpG) sites were methylated and cytosines in asymmetric (CpHpH) sites un-methylated. The PCR primers were selected following the guidelines described (Clark & Frommer, 1997). In selecting PCR

primers, regions containing symmetric sites were avoided and when this was not possible, a G residue was used instead of the C in the primers. The primers were targeted against unmethylated DNA, so that a lack of methylation in the result would not be attributed to the failure of bisulfite conversion. Amplification of the 35S:C4 transgene, including 98bp of the 35S promoter and complete *C4* ORF, was carried out by nested PCR in two consecutive reactions. The primer pair P24 and P25 (Table 2.2), was used in the first reaction and the primer pair P24 and P26 (Table 2.2), was used in the second reaction. The primer pair P27 and P28 (Table 2.2), was used to amplify a part of the intergenic region (IR; TLCV co-ordinates from 2706bp to 2614bp) and a part of *C1* ORF (TLCV co-ordinates from 2614bp to 2140bp) overlapping the complete sequence of *C4* ORF (TLCV co-ordinates from 2464bp to 2156bp). PCR was carried out as described (Grunau et al., 2000). PCR products were separated by electrophoresis in a 1% agarose gel, purified using the QIAquick gel extraction kit (Qiagen), ligated into pGEM-T-Easy (Promega) and introduced into *E. coli* XL1-Blue cells. Plasmid DNA was prepared from *E. coli* using a QIAGEN Spin Miniprep kit (QIAgene) and cloned DNA sequenced using ABI Prism BigDye (Perkin Elmer) with T7 or SP6 primers.

6.2.7 Statistical analysis.

Statistical comparisons of methylation data from 35S:C4 transgene and TLCV *C4* coding region and upstream non-coding region (nucleotides 2706-2465 of the intergenic region) were done by individual unpaired *t*-tests using the Graphpad Instant statistical software package. Due to unequal variances in asymmetric cytosine methylation in TLCV infected and healthy 35S:C4 transgenic plants, *t*-tests were performed on the log transformed data.

6.3 Results

6.3.1 A TLCV-derived transgene is silenced upon the viral infection.

Previous observations in our laboratory have suggested that transgenic plants expressing a range of TLCV-derived gene constructs in sense or anti-sense orientations have failed to protect tobacco or tomato plants from viral infections (Dry, et al., unpublished data). Recent experiments in our group have shown that TLCV infection is delayed but not prevented in *C2* hair pin (*C2*hp) transgenic plants designed to produce high levels of siRNAs specific to C2 ORF from TLCV (Bian et al., 2006). These results suggest that the TLCV-specific transgene in those transgenic plants becomes inactive after TLCV infection. To test whether the transgenes become inactivated, the fate of a TLCV-derived transgene was determined after virus infection. Tobacco plants containing the *C4* ORF under the control of CaMV 35S promoter (35S:C4) (Krake et al., 1998) were used because they express a severe phenotype that can serve as a marker for monitoring RNA silencing. The 35S:C4 phenotype includes curling of leaf margins, twisting of stems and mosaic patterns (Fig. 6.1A, panel 1). These are distinct from the mild symptoms caused by TLCV in tobacco (Fig. 6.1A, panel 4). After inoculation of the 35S:C4 plants in triplicate with TLCV, the typical 35S:C4 phenotypes disappeared gradually in all the plants. At 60 dpi, TLCV-infected 35S:C4 plants (Fig. 6.1A, panel 2) showed typical mild symptoms present on TLCV-infected non-transgenic plants (Fig. 6.1A, panel 4). The TLCV symptoms persisted and developed on the newly growing parts of transgenic plants. The presence of TLCV in transgenic and non-transgenic tobacco plants was confirmed by southern analysis (Fig. 6.1B). These results suggested that expression of the virus-derived 35S:C4 transgene might have been silenced following TLCV infection.

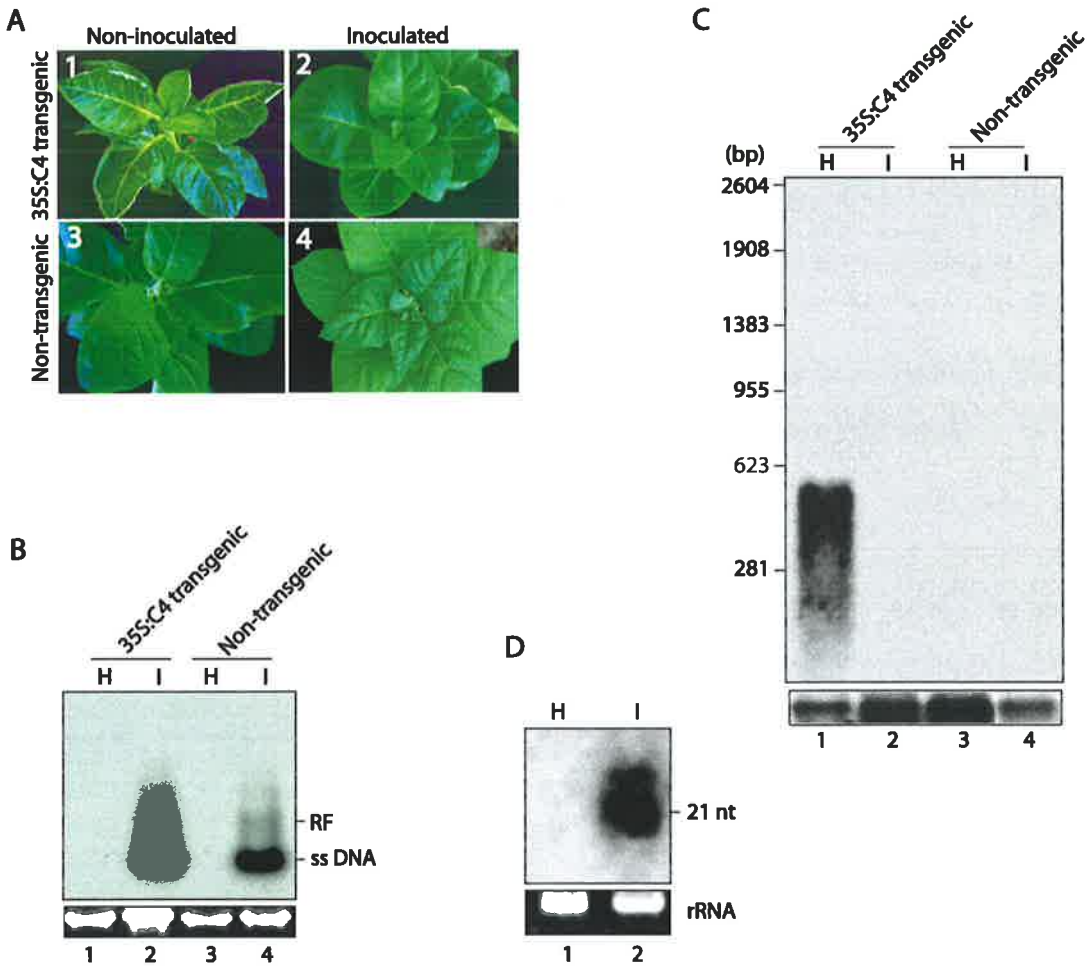


Figure 6.1. TLCV infection results in the silencing of a transgene homologous to the viral DNA. **(A)** Showing the C4 phenotype: (panel 1) Healthy 35S:C4, (panel 2) TLCV-infected C4 transgenic, (panel 3) healthy non-transgenic and (panel 4) TLCV-infected non-transgenic tobacco plants. Photographs were taken at 60 dpi. **(B)** Southern blot analysis of DNA extracted from healthy (H) or TLCV-inoculated (I) 35S:C4 transgenic and non-transgenic tobacco plants. The positions of replicative form (RF) and single-stranded (ss) DNA are shown. In the bottom panel, genomic DNA is shown as an indication of the loading levels. **(C)** Northern blot analysis of mRNA extracted from C4 transgenic and from non-transgenic tobacco leaves. Messenger RNA was extracted from either healthy (H) or TLCV-infected (I) leaves at 60 dpi. In the bottom panel, the membrane was stripped and reprobed for ubiquitin (Ubi) mRNA to provide an indication of RNA loading levels. **(D)** Northern blot analysis showing siRNA in TLCV-infected (I) *N. benthamiana* (lane 2). siRNA was not detected in a healthy (H) plant. RNA samples (5 μg) were separated by electrophoresis in a 15% denaturing polyacrylamide gel and transferred to a membrane. Hybridization was done with an in vitro transcribed, ^{32}P-labelled TLCV *C2* RNA probe. Ethidium bromide staining of the ribosomal RNA (rRNA) is shown as an indication of loading. The position of 21 mer oligonucleotides is shown.

To examine whether the change in 35S:C4 phenotype was due to RNA silencing, the level of *C4* transcript expressed from the 35S:C4 transgene was analysed before and after infection of the plants with TLCV. Northern blot analysis was carried out with a poly A^+ RNA preparation to avoid hybridization interference by TLCV DNA in infected samples. The *C4* transcript was readily detectable in non-inoculated 35S:C4 transgenic plants (Fig. 6.1C, lane 1). On the contrary, the transcript was not detected in the 35S:C4 transgenic plants at 60 dpi with TLCV (Fig. 6.1C, lane 2), or in healthy non-transgenic tobacco (Fig. 6.1C, lane 3). It was noted that the *C4* transcript expressed from replicating TLCV in non-transgenic tobacco plants was below the level of detection by the northern hybridization technique (Fig. 6.1C, lane 4). These results indicate that the 35S:C4 transgene was silenced following TLCV infection. Furthermore, TLCV-specific siRNAs were detected in TLCV infected plants (Fig. 6.1D) suggesting their role in transgene silencing. Previously it was shown in this laboratory, by nuclear run on assays, that the silencing of a transgene carrying homology to TLCV DNA is caused by virus induced transcriptional gene silencing (Seemanpillai et al., 2003). The silencing of the *C4* transgene is also likely to be due to TGS.

6.3.2 Tissue-specific replication of TLCV induces systemic silencing of the 35S:C4 transgene.

To investigate virus-induced gene silencing at the cellular level, in situ hybridization experiments were carried out with non-inoculated and TLCV-infected 35S:C4 transgenic tobacco leaf tissues, using fluorescent probes. Following hybridization with the *C4* virion-sense probe (C4-v), strong fluorescent output was observed in all cell types including phloem and mesophyll of non-inoculated 35S:C4 transgenic plants (Fig. 6.2B), indicating that there was constitutive expression of the *C4* transcripts from the

35S:C4 gene. When the sections of the TLCV-infected 35S:C4 transgenic plants were hybridized with the same probe, fluorescent signals were observed only in phloem-associated cells and were restricted to nuclei (Fig. 6.2C). These complementary-sense signals presumably represent the replicative form of TLCV DNA in the nucleus or *C4* transcripts, to which the C4-v probe can hybridize. The presence of viral nucleic acids in these cells was confirmed by probing the serial sections immediately adjacent to that shown in Fig. 6.2C with a digoxigenin-labelled complementary-sense probe from the *V2* ORF (V2-c). Strong colourimetric signals developed in the vascular cells indicating the presence of virion-sense DNA and transcripts (Fig. 6.2D) in both cytoplasm and the nucleus. In the same experiment, hybridization signals were not detected in healthy, non-transgenic leaves of tobacco (Fig. 6.2A). Moreover, vascular-associated accumulation of viral DNA and transcripts in non-transgenic tobacco (Fig. 6.2E) was found to be similar to that of transgenic tobacco (Fig. 6.2D). These results confirm that TLCV is phloem-specific (chapter 4) and causes a systemic silencing response in non-vascular cells. The un-interrupted replication of TLCV in vascular cells indicates that functional viral transcripts are produced in TLCV-infected phloem cells of silenced plants.

6.3.3 The *C4* gene in transgenic plants and in TLCV RF DNA is heavily methylated.

Previously, TLCV infection has been found to induce methylation of its homologous promoter sequence in transgenic plants (Seemanpillai et al., 2003). It was questioned whether the silencing of the *C4* gene encoded by the transgene and by the virus was linked to DNA methylation. Methylation status of the 35S:C4 transgene following TLCV infection and the *C4* region in TLCV RF DNA was determined by bisulfite-

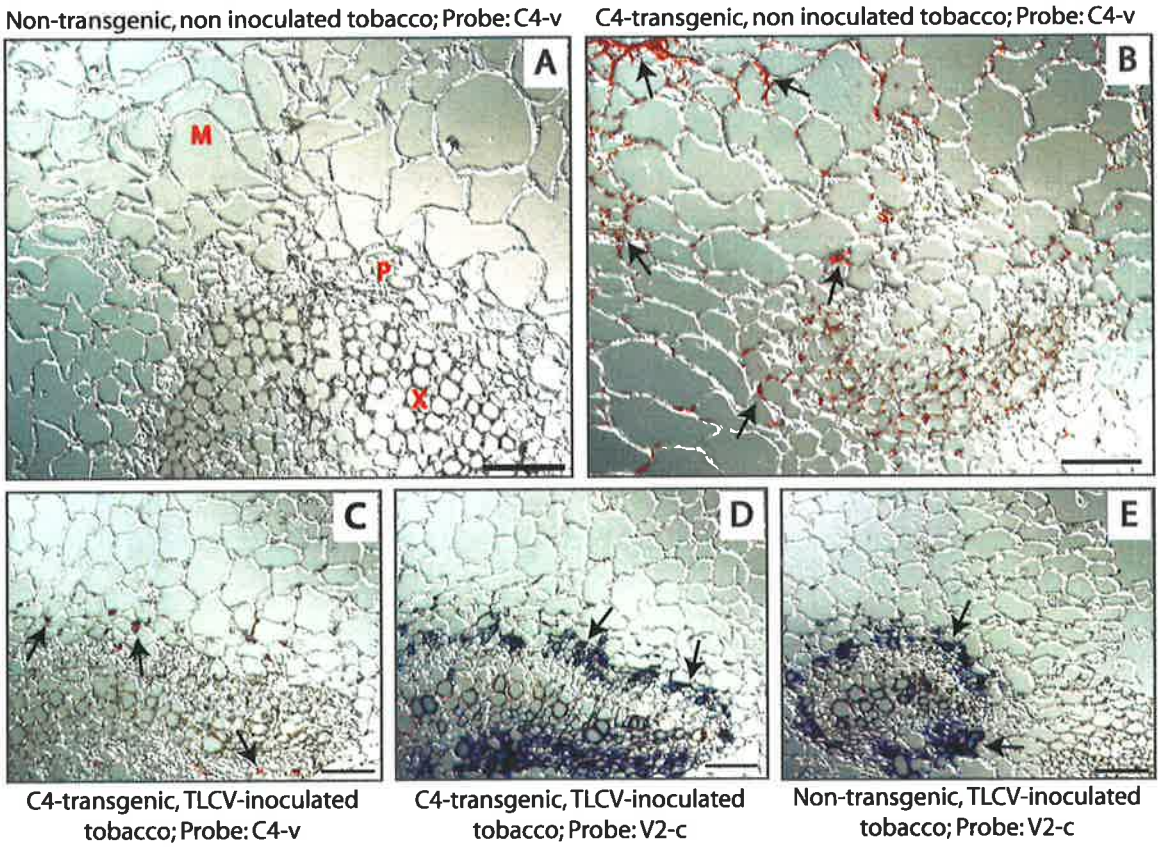


Figure 6.2. In situ hybridization showing TLCV-induced silencing of the *35S:C4* transgene. The sections were derived from healthy, non-transgenic tobacco **(A)**, healthy transgenic tobacco **(B)**, TLCV-infected transgenic tobacco **(C and D)** and TLCV-infected non-transgenic tobacco plants **(E)**. The probes were fluorescein-labelled, virion-sense ssRNA from the *C4* ORF **(A-C)** and digoxigenin-labelled, complementary-sense ssRNA from the *V2* ORF **(D and E)**. The sections were examined using differential interference contrast optics. Hybridization signals are indicated by arrows. M = mesophyll, X = xylem and P = phloem. Bars = 100 μm.

sequencing (Clark & Frommer, 1997). Total genomic DNA was extracted from non-inoculated 35S:C4 transgenic, TLCV-infected 35S:C4 transgenic and TLCV-infected non-transgenic tobacco leaves at 60 dpi and subjected to methylation sequencing. In the procedure, bisulfite treatment converts un-methylated cytosines to uracil while methylated cytosine remains unchanged. The converted DNA is then cloned and sequenced. To ascertain DNA conversion, the methylation status of the tobacco *5S* rRNA gene was determined (Fulnecek & Matyasek, 1998) in bisulfite treated samples. The three samples were found to have very similar methylation status in the *5S* rRNA gene: all of the 11 symmetric cytosines were methylated and all of the 15 asymmetric cytosines were un-methylated (data not shown). Thus, a total of 42% cytosines were methylated, a rate consistent with the previous report of tobacco *5S* rDNA methylation (Fulnecek & Matyasek, 1998).

The region of the 35S promoter and *C4* ORF was cloned using bisulfite-treated total DNA from TLCV inoculated and non-inoculated 35S:C4 transgenic plants. Five individual clones from each sample were sequenced and the sequences were aligned. A high level (86-92%) of symmetric cytosine methylation was observed in both TLCV-infected and non-infected 35S:C4 transgenic plants (Table 6.1, Fig. 6.3A). The difference in the levels of symmetric cytosine methylation between infected and healthy transgenic plants was not statistically significant (Fig. 6.3A). Contrary to the levels of symmetric cytosine methylation, there was a statistically significant ($p=0.032$) increase in asymmetric cytosine methylation of the transgene after TLCV infection. In the non-inoculated plants, only 6.2% of asymmetric cytosines were methylated (Table 6.1, Fig. 6.3A), whereas in infected 35S:C4 transgenic plants, 39.3% of asymmetric cytosines were methylated (Table 6.1, Fig. 6.3A). Cytosine methylation was observed not only in

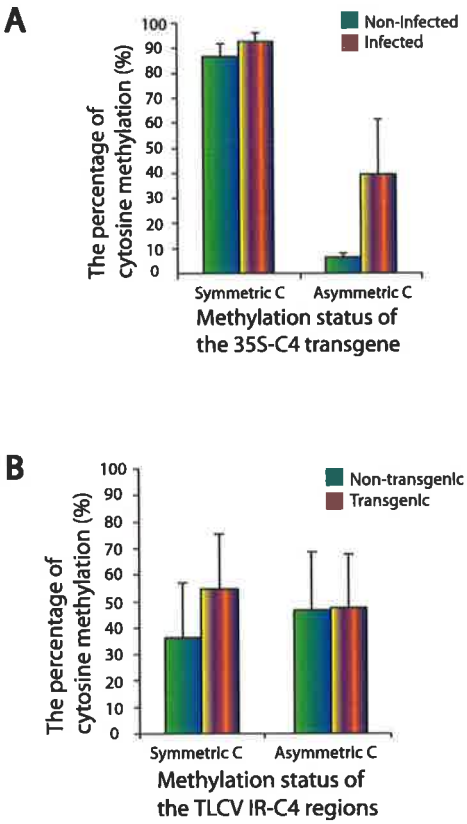


Figure 6.3. Analysis of cytosine methylation of the *35S:C4* trangene and TLCV IR-C1 region. Genomic DNA was extracted, treated with bisulfite, amplified by PCR and cloned as described in Materials and Methods. **(A)** The percentage of methylated symmetric and asymmetric cytosines in the *35S* promoter and the *C4* ORF was calculated separately for each individual clone. The averages and standard deviation calculated from the five independent clones analyzed are shown. **(B)** The percentage of methylated symmetric and asymmetric cytosines in the IR and the *C4* ORF was calculated separately for each individual clone. The averages and standard deviation calculated from the four independent clones analyzed are shown.

the *C4* coding region which has homology with TLCV DNA but also in the 35S promoter region with no homology to the viral genome (Table 6.1).

Given that geminivirus replication occurs in the host cell nuclei (chapter 4) (Rasheed et al., 2006; Rojas et al., 2005), it was questioned whether TLCV DNA was also subjected to methylation by the host. A fragment of DNA containing *C4* ORF was amplified by PCR from the bisulfite-treated genomic DNA of TLCV-infected 35S:C4 transgenic and non-transgenic tobacco plants. The use of TLCV-specific primers outside the region present in the transgene ensured that the transgene was not amplified. PCR products were cloned and four individual clones were sequenced from each sample and aligned. Interestingly, the TLCV DNA region sequenced contained methylated cytosines at both symmetric and asymmetric positions. The levels of cytosine methylation at either symmetric or asymmetric sites of TLCV-infected transgenic and non-transgenic plants ranged between 36 and 54% (Table 6.1, Fig. 6.3B). These differences were not statistically significant. These results indicated that TLCV DNA was methylated during its infection cycle, although at significantly lower levels compared to the transgene, and that the methylation of TLCV RF DNA was independent of the presence of the 35S:C4 transgene. The level of cytosine methylation was also noted in a 50 nt segment of the non-coding intergenic region, which was present in the methylation sequencing data, and was close to the methylation level of *C4* ORF (48% and 53% respectively). However there was no evidence to suggest that TLCV DNA methylation was preferentially directed to either the coding region or non-coding region.

Table 6.1. The rates of methylation of 35S:*C4* transgene and TLCV *C1*-IR

	Regions	Plants	Cytosine methylation (%)	
			Symmetric	**Asymmetric**
Transgene	35S Promoter	**I**[a]	94.3	36.7
		H[b]	89.7	7
	C4 ORF	**I**	80	46.9
		H	64	5.2
	Combined	**I**	92.5	39.3
		H	86.5	6.4
TLCV RF	*C1*-IR[c]	**T**[d]	52.6	48.9
		NT[e]	31.9	33.7
	C4 ORF	**T**	64.3	46.2
		NT	53.6	58.2
	Combined	**T**	54.9	47.5
		NT	36.1	45.9

[a] TLCV-infected tobacco plants
[b] Healthy tobacco plants
[c] A segment of *C1* and intergenic region containing the entire overlapping *C4* ORF
[d] 35S:C4 transgenic tobacco plants
[e] Non-transgenic tobacco plants

Comparisons of methylation data presented in Fig. 6.3A and 6.3B revealed a standard deviation associated with TLCV-infection. The possible significance of this observation is discussed below.

6.4 Discussion

RNA silencing is an effective defence tool against RNA viruses (Hamilton & Baulcombe, 1999; Voinnet, 2001). However, application of this strategy against geminiviruses has produced inconsistent results. This is probably the reason why numerous attempts have been made to develop transgenic resistance against geminiviruses (Covey & Al-Kaff, 2000). For example, resistance against TYLCV considered originally to be mediated by truncated Rep (Brunetti et al., 1997; Noris et al., 1996) was found to involve RNA silencing (Lucioli et al., 2003) and to be overcome by the virus (Noris et al., 2004). More recently, several variations of the TYLCV Rep gene constructs were introduced into the tomato genome (Yang et al., 2004). The plants produced a range of responses to the virus, from susceptibility with some constructs to resistance with others. Within transgenic lines showing resistance, a proportion of the plants became infected, despite the presence of the transgene.

More successful results have been reported for ACMV. Rep gene constructs produced variable but significant resistance to the virus, and the resistance was suggested to involve RNA silencing rather than protein interference. However, this resistance was only effective against distinct begomovirus species infecting cassava (Chellappan et al., 2004a).

Consistent with previous reports, attempts in our lab to develop resistance against TLCV using a range of gene constructs have been unsuccessful. Recently work in our group has shown that the *C2* hp transgenic plants produced a significant level of siRNA. Nevertheless they only exhibited delayed symptoms and resistance was overcome by TLCV infection (Bian et al., 2006).

Evidence supporting the ability of geminiviruses to escape RNA silencing also comes from studies using these viruses as silencing vectors. A number of geminivirus-host systems have been used to demonstrate the silencing of introduced or endogenous plant genes by episomal replication of the vectors (Atkinson et al., 1998; Fofana et al., 2004; Peele et al., 2001; Tang & Leisner, 1998; Tao & Zhou, 2004). These systems share a common feature with those targeted for virus-derived resistance. In these cases the host plants share DNA sequence homology with the virus involved. In the cases of geminivirus-induced gene silencing where virus replicates in the silenced plant (Atkinson et al., 1998; Kjemtrup et al., 1998; Peele et al., 2001), the silencing of a plant gene does not prevent the replication of homologous virus. In line with these findings, it has been observed that TLCV promoter-driven GUS transgenes become silenced when the plants are infected with the homologous virus, while the replication of TLCV itself is not affected significantly (Seemanpillai et al., 2003). Likewise, a severe phenotype induced by the βC1 protein of a geminiviral satellite DNA was completely silenced upon inoculation of the plants with the satellite and the helper virus while virus replication continued (Saeed et al., 2005).

The inconsistencies in reports of transgenic resistance and lack of success with the *C2* hp transgenic plants in our group (Bian et al., 2006) led us to speculate that a TLCV-

derived transgene may become silenced upon infection with TLCV. To examine this possibility, we analysed the fate of a 35S promoter-driven TLCV *C4* transgene following infection.

Analysis of 35S:C4 transgenic plants revealed high levels of symmetric cytosine methylation of the transgene, irrespective of whether these plants were infected with TLCV or were healthy (Fig. 6.3A). This is in agreement with previous results (Seemanpillai et al., 2003) that TLCV promoter-driven GUS transgenes have high levels of methylation of symmetric cytosines, and with the report that about 80% of symmetric cytosines in higher plant genomic DNA are methylated (Gruenbaum et al., 1981). It has been suggested that methylation of symmetric cytosines may be independent of their transcriptional activity (Pena et al., 2004).

Contrary to the methylation of symmetric cytosines, methylation of asymmetric cytosines in the 35S:C4 transgene was quite low but increased significantly following TLCV infection of the plants. Consistent with this finding, VIGS by both an RNA virus (Jones et al., 2001) and a DNA virus (Rodman et al., 2002) has been reported to be associated with methylated asymmetric cytosines, a process linked to gene suppression (Dieguez et al., 1997). It therefore appears likely that the observed silencing of the *C4* transgene is linked to the enhanced level of asymmetric cytosine methylation following TLCV infection. The asymmetric cytosine methylation may be a host response to TLCV infection which targets both the viral RF DNA (discussed below) and its transgene homologue.

Interestingly, asymmetric cytosine hypermethylation of the *C4* coding region of transgenic plants infected with TLCV extended to the 35S promoter (98 bp analysed). Spread of methylation to transcribed sequences not homologous to the viral genome has been reported upon RNA virus infection (Jones et al., 1999; Van Houdt et al., 2003). Since the transgene of 35S:C4 transgenic plants contained a tandem-repeat of the 35S promoter, it is possible that a population of the transcripts was initiated from the first promoter element and therefore contained the promoter sequences.

A high level methylation of both symmetric and asymmetric cytosines was also observed in TLCV RF DNA. In contrast to this finding, methylation analysis of TGMV DNA by methylation-sensitive restriction enzymes has shown that the viral dsDNA is not appreciably methylated at 13 restriction sites tested in the entire DNA molecule (Brough et al., 1992). The reason for this difference remains unknown but may relate to the analytical approaches utilized. The bisulfite DNA sequencing used in our study reveals methylated positions across the entire target sequences rather than only at restriction sites. With respect to the levels of methylation, TLCV DNA resembles the host plant genome, where high levels of most symmetric cytosines are methylated (Gruenbaum et al., 1981). Like the plant DNA, geminiviral DNA replicates in the cell nuclei and is organized in the form of minichromosomes (Pilartz & Jeske, 1992). It is therefore possible that TLCV DNA is subjected to the same process that results in high levels of symmetric cytosine methylation in plants.

The replication of TLCV in silenced plants indicates that the viral genes including complementary-sense genes, which are indispensable for viral DNA replication, are expressed. Various possibilities can be envisaged to explain how TLCV gene

expression can take place in a silenced background. The silencing process, although highly effective against the transgene (Fig. 6.1C), may be incomplete, leaving a small pool of viral transcripts that may be sufficient for TLCV replication. Alternatively, the silencing process may not be operating in the phloem cells where TLCV replication occurs (Fig. 6.2C). Such a possibility is plausible (Noris et al., 2004) because TLCV, like other begomoviruses, encodes a suppressor of RNA silencing (Selth et al., 2004). With TGMV, it has been found that the RNA silencing suppressor is only present in the virus infected cells (Wang et al., 2003). Here it has been shown that TLCV is restricted to phloem cells but the silencing of 35S:C4 transgene spreads systemically. This suggests that a silencing signal is produced in TLCV-infected cells and then spreads to other tissues. Therefore, the silencing process is functional in non-infected tissues where the TLCV-encoded suppressor protein is likely to be absent. Given the presence of various forms of viral DNA and viral transcripts in TLCV-infected cells, it is unclear whether the transgene homologue was expressed from TLCV DNA in infected cells.

TLCV DNA has been found to replicate by a combination of rolling circle replication (RCR) and recombination-dependent replication (RDR) (Alberter et al., 2005). RCR is a semi-conservative process resulting in the production of RF DNA (Saunders et al., 1991), Fig. 6] consisting of a *de novo* unmethylated strand and a pre-existing methylated strand (Fig. 6.4). In eukaryotic cells the pattern of methylation is rapidly copied from the pre-existing to the *de novo* strand by methyltransferases that recognize methylated CG dinucleotides (Holliday & Pugh, 1975; Smith et al., 1992). In contrast, both DNA strands produced by RDR are synthesized *de novo* and initially lack methylation. Although TLCV DNA showed a significant overall methylation, replication of TLCV DNA by RDR (Alberter et al., 2005) may give rise to a population

of unmethylated, transcriptionally active DNA (Fig. 6.4B) that may become methylated subsequently. Consistent with this idea, in-vitro methylated TGMV (Brough et al., 1992) and ACMV (Ermak et al., 1993) DNA have been reported to replicate with lower efficiencies in transfected protoplasts and to produce demethylated progenies (Brough et al., 1992; Ermak et al., 1993).

A high level of variation in the methylation levels of TLCV RF DNA was observed, as indicated by a relatively large standard deviation (Fig. 6.3B). Furthermore, the rate of symmetric cytosine methylation of TLCV RF DNA was considerably lower compared to the transgene (compare Fig. 6.3A and 6.3B). This is consistent with the suggestion that a proportion of TLCV DNA produced by RDR (Alberter et al., 2005) may not be an efficient target for symmetric cytosine methylation by methyl transferases. Data in Fig. 6.3A indicate that asymmetric cytosine methylation of the transgene was also variable in TLCV infected plants. The reason for this variation remains unknown, although it could be speculated that this variation is the consequence of a sequence specific process (eg siRNA mediated) that affects viral DNA methylation.

Assuming that the ability of TLCV to replicate in silenced plants is mediated by the lack of DNA methylation, it could be argued that this process is regulated by the viral C2 protein which is a suppressor of gene silencing (Selth et al., 2004; van et al., 2002; Vanitharani et al., 2004; Voinnet et al., 1999). Wang et al. (2003) have demonstrated that AL2 (also designated AC2 and C2) of geminiviruses interacts with adenosine kinase (ADK) and have suggested that AL2 inhibits ADK activity by interfering with DNA methylation. ADK activity has been shown to play an important role in *S*-adenosylmethionine-dependent transmethylation (Lecoq et al., 2001; Wang et al.,

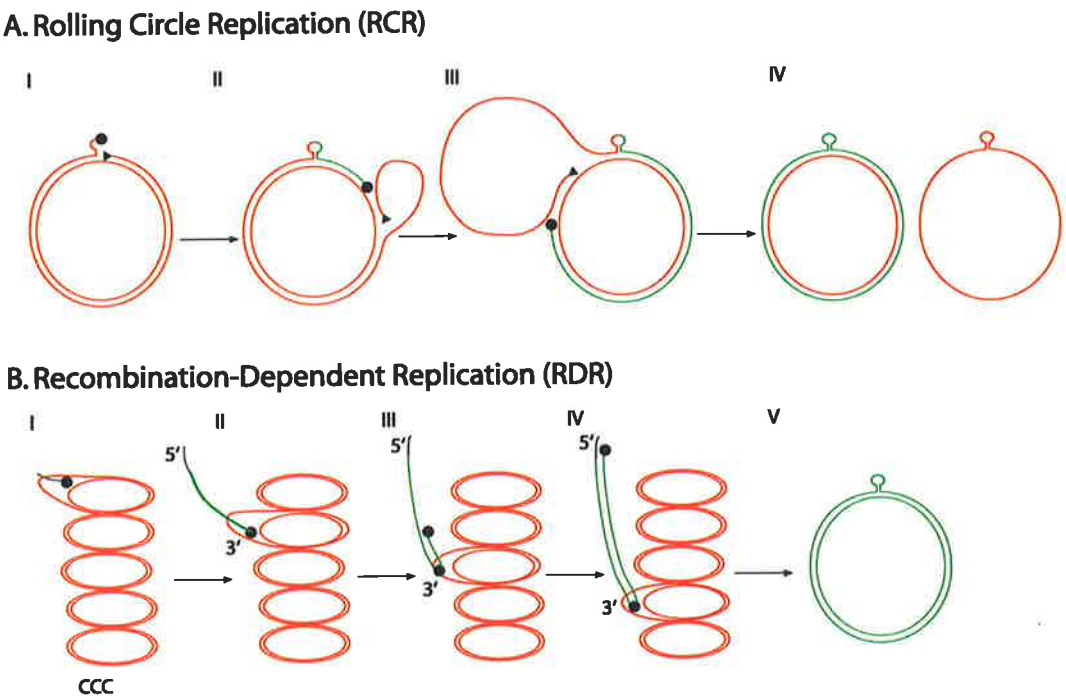


Figure 6.4. A model depicting methylation status of TLCV DNA produced by **(A)** rolling circle replication (RCR) and **(B)** by recombination-dependent replication (RDR). In RCR, dsDNA is methylated in one strand (red line) and is unmethylated in the other strand (green line). Both DNA strands produced by RDR are unmethylated (green lines). Roman numerals specify sequential steps in DNA replication. The figure has been re-drawn from published data (Jeske et al., 2001, Rigden et al., 1996).

2003). The finding of this study that TLCV RF DNA is highly methylated indicates that a C2 induced inhibition of methylation is not occurring in infected cells. It remains possible that a C2/ADK mediated inhibition of methylation would contribute to the generation of a small pool of unmethylated TLCV RF sufficient for viral transcription.

A high level of TLCV-specific siRNA was found in the virus infected *N. benthamiana* plants (Fig. 6.1D). It would therefore appear that TLCV has evolved the mechanisms to efficiently overcome the host silencing response. siRNA is known to mediate gene silencing through methylation of target sequences (Cao et al., 2003). It remains unclear whether TLCV DNA methylation is linked to the presence of virus specific siRNA.

The detection of geminivirus-specific siRNAs in infected hosts suggests that an RNA silencing process is triggered in response to DNA virus infection. These results indicate that TLCV has evolved the means of circumventing this silencing pathway. This may explain why the development of a virus-derived resistance against geminiviruses has provided inconsistent results while these viruses continue to pose a significant challenge to global agriculture.

Chapter 7 – CLCuMV associated satellite DNA β localizes with the helper TLCV in the phloem

7.1 Introduction

The term "satellite" was first used by Kassanis (1962) and now it defines viruses or nucleic acids that depend on a helper virus for their replication but lack appreciable sequence homology to the genome of the helper virus, and are dispensable for proliferation of the helper virus (Mayo et al., 2005). A large number of satellite RNAs associated with diverse groups of plant RNA viruses have been reported (Mayo et al., 2005). The first satellite of a DNA virus was isolated in this laboratory from tomato plants infected with TLCV (Dry et al., 1997). This circular satellite is a 682 nucleotide ssDNA molecule, has no putative ORFs and has little sequence homology with its helper virus except for a stem-loop containing the conserved TAATATTAC nonanucleotide typical of geminiviruses (section 1.1.4). The TLCV satellite is not required for TLCV replication and has no effect on symptom development but is dependent on the helper virus for its replication and encapsidation.

A related group of satellite DNA molecules, referred to as DNA β, has recently been found to be associated with monopartite begomoviruses (Briddon et al., 2003; Mansoor et al., 2003). In contrast with the TLCV satellite, DNA β may be essential for the induction of disease symptoms by the helper virus (Briddon et al., 2001; Jose & Usha, 2003; Saunders et al., 2000; Saunders et al., 2003). DNA β is approximately half the size (1.3-1.4 kb) of its helper virus DNA and has a highly conserved structure consisting of a single conserved complementary-sense ORF (designated *βC1*), an adenine rich

region, and a sequence highly conserved between all DNA βs, termed the satellite-conserved region (SCR) (Briddon et al., 2003). Recent studies have shown that the *βC1* gene encodes a protein which has a role in pathogenicity (Cui et al., 2004; Saeed et al., 2005; Saunders et al., 2004). Expression of the *βC1* gene either transiently with a PVX vector or stably in transgenic *N. benthamiana* plants, produced severe developmental abnormalities, vein-greening, and disorganized cell division (hyperplasia) in the vascular bundles (Saunders et al., 2004). The precise function of βC1 in pathogenesis is unknown, although it has been suggested that βC1 is either involved in suppression of gene silencing (Cui et al., 2005) or may have a role in facilitating the movement of monopartite begomoviruses (M. Saeed, unpublished results)

Following the identification of the DNA β molecule associated with AYVV (Saunders et al., 2000), these satellites have been found to be associated with the majority of monopartite begomoviruses (Mansoor et al., 2003). It has been shown that the AYVV associated DNA β can transreplicate with the DNA A of the bipartite *Sri Lankan cassava mosaic virus* (SLCMV) (Saunders et al., 2002). Likewise, Saeed et al. (2005) have demonstrated that DNA β associated with the monopartite CLCuMV can use TLCV as a helper virus. This possibly explains why begomovirus-DNA β disease complexes are widespread. This chapter describes experiments on the spatial distribution of DNA β and its helper viruses CLCuMV and TLCV at the tissue and cellular level.

7.2 Materials and methods

7.2.1 Plant materials.

Tobacco (*Nicotiana tabacum* cv. Samsun and *Nicotiana benthamiana*) and cotton (*Gossypium hirsutum*) were grown in the glasshouse at 25-30°C. Transgenic tobacco lines carrying either a CaMV 35S promoter-driven *C4* ORF cassette (Krake et al., 1998) or a head-to-tail dimeric construct of CLCuMV DNA β (Saeed et al., 2005) were grown under PC2 containment.

7.2.2 Infectivity assays.

Plants at the 4-5 leaf stage were inoculated with an overnight culture of *A. tumefaciens* (strain C58) containing infectious viral clones (Table 7.1). Virus content of leaf was determined by sampling 50-100 mg tissue pieces, extracting with 0.5 M NaOH at a ratio of 4 µl per 1 mg tissue, and identifying viral DNA by dot-blot analysis (Stonor et al., 2003).

7.2.3 In situ hybridization.

DNA templates for the generation of RNA probes were amplified by the polymerase chain reaction (PCR) using primer pairs P5/P6, P29/P30 and P31/P32 (Table 2.2). RNA probes labelled with fluorescein-12-UTP and digoxigenin-11-dUTP were prepared using Fluorescein or DIG RNA Labelling Mix respectively (Roche Diagnostics, Castle Hill, Australia) as described in section 3.2.5.

Preparation of tissue sections, hybridization and detection of DIG- and fluorescein-labelled probes was carried out as described in section 3.2.

Table 7.1. Infectious clones of CLCuV, DNA β, TLCV, TGMV and TYLCSV

Plasmid Designation	Construct	Source
pBin CLCuV	1.5 mer	(Briddon et al., 2000)
pBin DNA β	2.0 mer	(Briddon et al., 2001)
pBin TLCV	2.0 mer	(Dry et al., 1993)
pBin TGMV A	2.0 mer	(von Arnim & Stanley, 1992)
pBin TGMV B	2.0 mer	(von Arnim & Stanley, 1992)
pBin TYLCSV	2.0 mer	(Kheyr-Pour et al., 1991)

7.3 Results

7.3.1 *Cotton leaf curl Multan virus* and its satellite DNA β are co-localized in the phloem.

Tissue tropism of CLCuMV and its satellite DNA β was studied by in situ hybridization using complementary-sense RNA probes corresponding to *C1* and *βC1* ORFs respectively. These probes were designed to detect viral DNA, not the transcripts. The genome organization, position and orientation of the probes of both CLCuMV and satellite DNA β are shown in Figure 7.1. Both CLCuMV and DNA β were found in the vascular tissues of systemically infected cotton leaves and signals were not detected in other cell types outside the vascular tissues (Fig. 7.2A and 7.2B). Virus specific signals were found in fewer vascular cells compared to signals detected in sections derived from *N. benthamiana* co-infected with TLCV and DNA β (compare Fig. 7.2A and 7.2B with 7.2C and 7.2D). Hybridization signals were not found in the sections of uninfected tissues and hybridization with heterologous probes (data not shown) as described in section 4.3.1.

7.3.2 CLCuMV DNA β replicates with TLCV and localizes within the same phloem cells.

It has been shown that CLCuMV DNA β can transreplicate with TLCV (Saeed et al., 2005). To observe that interaction at the cellular level, in situ hybridization was performed on sections taken from *N. benthamiana* co-infected with TLCV and DNA β. DNA β was found in the vascular cells containing TLCV although DNA β-specific hybridization signals were not as strong as TLCV (Fig. 7.2C, 7.2D, 7.2F and 7.2G). To identify DNA β and TLCV signals in the same cell, fluorescein-labelled TLCV-specific probe and DIG-labelled DNA β-specific probes were used. Dual in situ hybridization

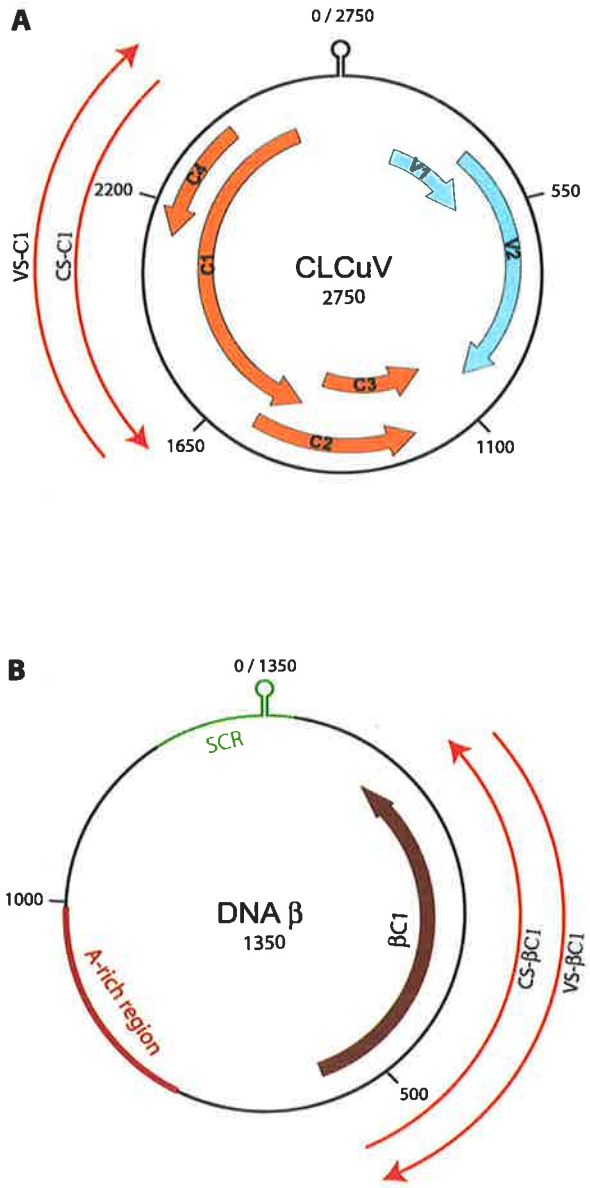


Figure 7.1. Genome organization of CLCuV (A) and DNA β (B) molecules showing the position and orientation of strand-specific RNA probes (thin arrows). **(A)** ORFs on the virion-sense (clockwise) strand and the complementary-sense (anticlockwise) strand are shown by arrows. The position of the conserved stem-loop structure is marked (reproduced from Mansoor et al., 2003). **(B)** The position and orientation of the conserved β*C1* ORF is shown as an arrow. The relative position of the satellite conserved region (SCR) and A-rich region are coloured (reproduced from Briddon et al., 2003). VS = virion sense, CS = complementary sense

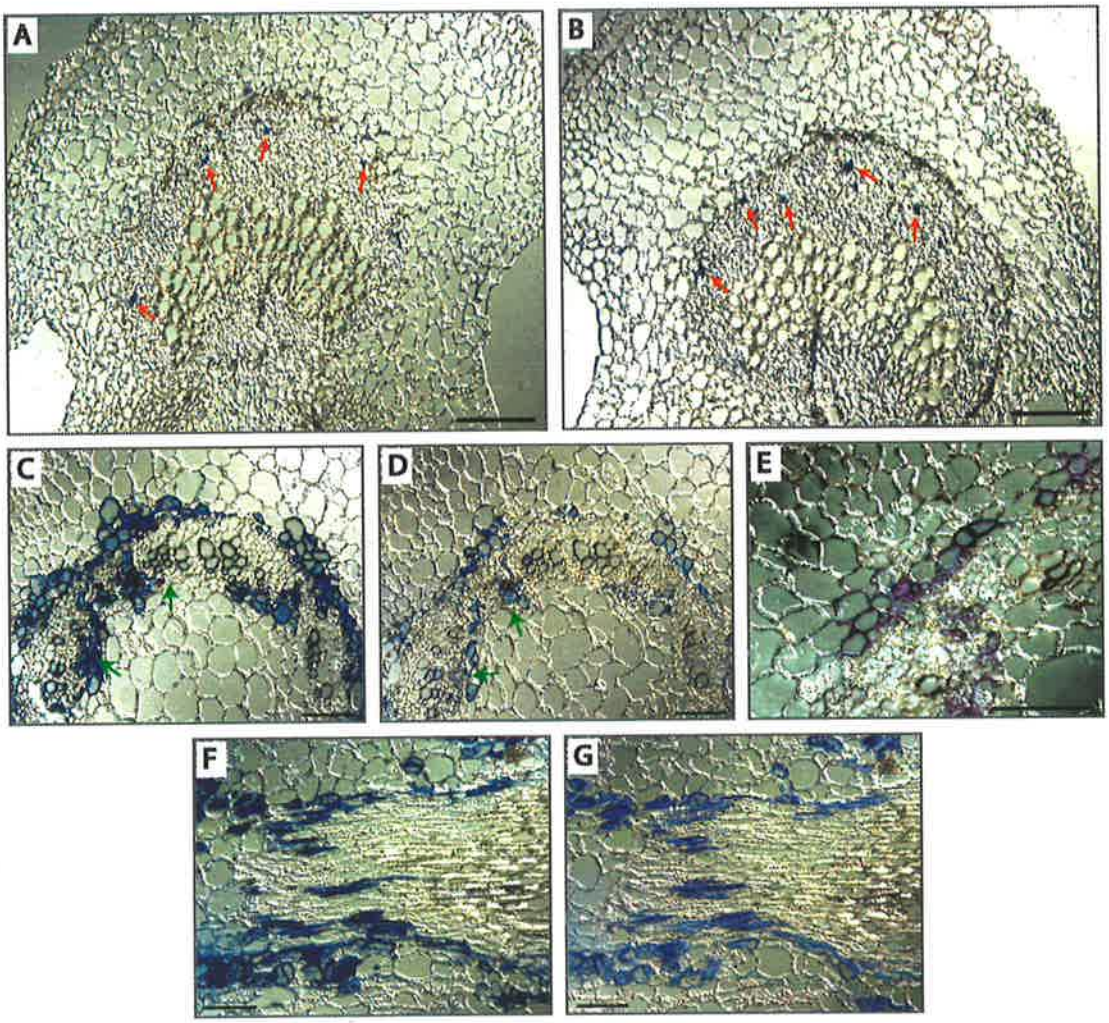


Figure 7.2. In situ localization of satellite DNA β with its helper viruses CLCuMV and TLCV. Tissue sections of cotton co-infected with CLCuV and DNA β (A-B) and *N. benthamiana* co-infected with TLCV and DNA β (C-G). Sections were hybridized with digoxigenin (DIG) labelled complementary-sense ssRNA from the β*C1* ORF (B, D, E and G) and from the *C1* ORFs of CLCuMV (A), DIG-labelled (C and F) or fluorescein-labelled (E) complementary-sense ssRNA from the *C4* ORF of TLCV. Sections A-E were transverse, F and G were longitudinal. Red arrows in A and B represent the hybridization signals and green arrows indicate groups of hyperplastic cells. Bars = 100 μm.

exhibited a distinct purple chromogenic output due to the masking of red TLCV signals by the blue DNA β signal (Fig. 7.2E), and confirmed that DNA β was detectable in almost every cell that accumulated TLCV.

Saeed et al. (2005) have reported that DNA β was rescued from a dimeric β transgenic tobacco plant (2β tobacco) upon TLCV infection. DNA β molecules should be present in all cell types of both vascular and non-vascular tissues of 2β transgenic tobacco. To investigate the localization pattern of TLCV and rescued DNA β molecules, in situ hybridization was done on sections of 2β transgenic tobacco infected with TLCV. Hybridization signals were found in the vascular cells of serial sections derived from 2β transgenic tobacco infected with TLCV using a complementary-sense RNA probe either specific to DNA β (Fig. 7.3B) or to TLCV (Fig. 7.3C). Following hybridization with the DNA β-specific probe designed to detect *βC1* specific RNA transcripts (virion-sense RNA probe from the *βC1* ORF), chromogenic signals were not detected in the section from the non-inoculated plant suggesting that transcripts if present are probably below detection level (Fig. 7.3A). This is consistent with the earlier observation that accumulation of *βC1* mRNA is low in 2β transgenic tobacco (Saeed et al., 2005). This result demonstrated that the DNA β molecule was released from the chromosome of 2β transgenic tobacco in the TLCV containing phloem cells and was amplified during replication of the helper virus.

7.3.4 Expression of *βC1* of DNA β and *C4* of TLCV in transgenic plants induces abnormal cell division.

Observation of tissue sections derived from TLCV and DNA β infected tissues occasionally revealed abnormal cell growth (Fig. 7.2C, 7.2D and 7.2E, green arrows). It

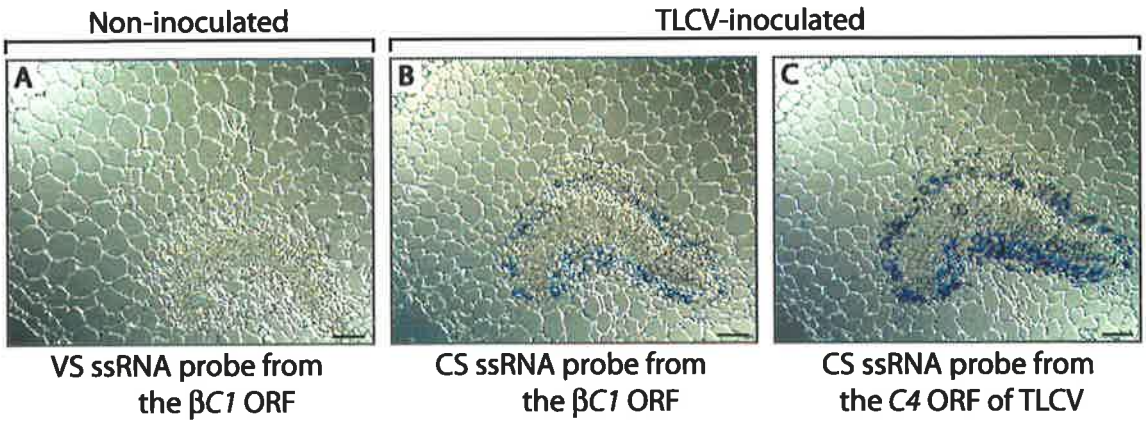


Figure 7.3. Rescue of DNA β in phloem cells upon TLCV infection of 2β transgenic plants. Transverse sections of transgenic plants containing complete DNA β molecule (A-C). Section shown in A was hybridized to detect βC1 mRNA, in B to detect DNA β and in C to detect TLCV. Viral infections and type of probe used are indicated. VS = virion sense, CS = complementary sense. Bars = 100 μm.

has been shown that transgenic tobacco plants expressing either the *βC1* gene from DNA β (Saeed et al., 2005) or the *C4* gene from TLCV (Krake et al., 1998) show growth abnormalities and severe phenotypic symptoms. When these abnormalities in βC1 and C4 transgenic plants were examined at the cellular level, large clusters of disorganized small cells were frequently observed to be associated with the vascular tissue (Fig. 7.4A and 7.4B). These abnormal cell structures were not found outside the vasculature. Similar hyperplastic cells were not observed in non-transgenic tobacco (Fig. 7.4C).

7.4 Discussion

To date no monopartite mesophyll invasive begomovirus has been identified. So far more than one hundred satellite DNA β molecules have been characterized (Briddon & Stanley, 2006), all associated with monopartite begomoviruses (Rojas et al., 2005). Co-localization of DNA β with its helper virus in phloem cells is reported for the first time in this study. It has been suggested that DNA B of bipartite begomoviruses may be involved in mesophyll invasion (Rojas et al., 2005). Satellite DNA β functionally resembles DNA B molecule of bipartite begomoviruses and hence has been given the name DNA β (Briddon & Stanley, 2006). Therefore, it was proposed that, like some of DNA B components of bipartite begomoviruses, DNA β may have the capability of mesophyll invasion. However, the results of this study do not support this assumption. Experiments in this laboratory have shown that CLCuMV DNA β promoter has phloem specific activity (Omid Gandomani, unpublished results). Likewise, phloem specific promoter activity of another DNA β associated with *Tomato yellow leaf curl China virus* (TYLCCNV) has been reported (Guan & Zhou, 2006). This suggests that the

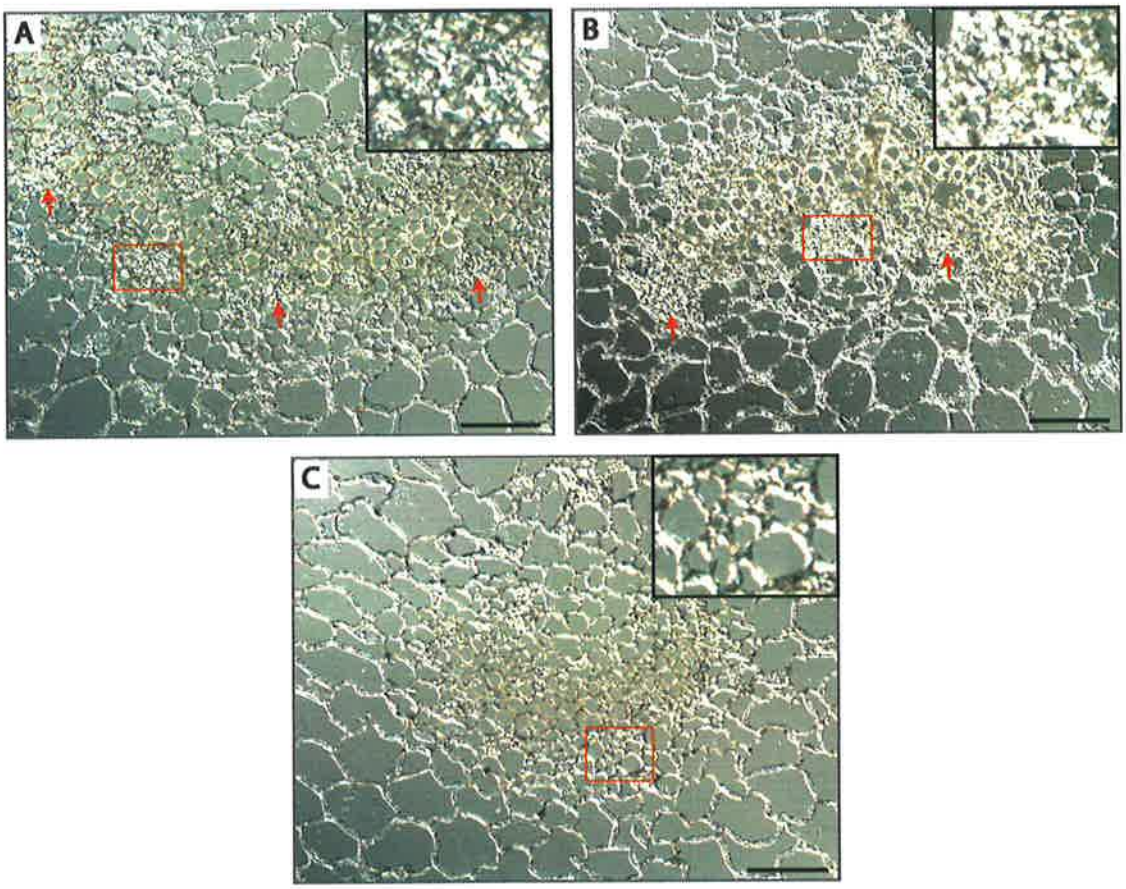


Figure 7.4. Hyperplastic cells in *C4* and 2β transgenic plants. Transverse sections of transgenic plants expressing the *C4* ORF of TLCV (A) and the dimeric DNA β molecule (B) showing groups of hyperplastic cells indicated by red arrows. A section from a non-transgenic plant is shown in C.The area inside the red box is enlarged in the inset in respective panels. Bars = 100 μm.

phloem specificity of DNA β is not solely determined by the tissue specificity of its helper virus.

The evolutionary origin of DNA β is not known. It has been assumed that DNA β may have evolved from a pre-existing component by association with a monopartite begomovirus (Mansoor et al., 2003). Another suggestion is that recombination may have resulted in the generation of a DNA B component of a bipartite begomovirus from a monopartite begomovirus (Saunders et al., 2002). A recombination between DNA A and DNA β of AYVV has been reported to produce a viable DNA β recombinant (Saunders et al., 2001a). A prerequisite for such a recombination is the replication of different viruses within the same cell. The result of this study suggests that phloem specificity may provide the opportunity for such an evolutionary process.

The occurrence of hyperplastic cells in 2β transgenic plants suggested that βC1 may have a role in reprogramming the cell division of infected cells, perhaps to facilitate the replication of their genome. Hyperplastic cells were also observed in C4 transgenic plants. This is consistent with the previous reports that the expression of either the *C4* gene of BCTV or the *βC1* of AYVV DNA β in transgenic *N. benthamiana* plants induces hyperplasia (Latham et al., 1997; Saunders et al., 2004). Further study is required to analyze the direct role of these proteins in cell signalling mechanisms and to identify host factors which may interact with these proteins, to regulate the cell cycle. It seems possible that gene products of both CLCuMV βC1 and TLCV C4 are functionally analogous. In transgenic plants, C4 and βC1 have the ability to induce disease symptoms (Krake et al., 1998; Saeed et al., 2005). In addition, C4 (Chapter 5) and βC1 (M. Saeed, unpublished results) have been shown to have a role in viral movement. This

is consistent with C4 of TYLCV which has been implicated in virus movement (Rojas et al., 2001). It has been suggested that gene silencing suppressors induce pathogenic effects in plants (Voinnet et al., 1999). Consistent with this, βC1 associated with TYLCCNV (Cui et al., 2005) and AC4 of SLCMV (Vanitharani et al., 2004) function as a suppressor of gene silencing. As for other geminivirus proteins, it is likely that βC1 and C4 have a multifunctional role.

CLCuMV DNA β has been found to spread systemically with monopartite phloem specific begomoviruses like TLCV (this study) and TYLCV (data not shown) and this can be coupled with bipartite ToLCNDV (M. Saeed, unpublished results) which are not sap-transmissible and are therefore assumed to be phloem-specific. The study of DNA β association was extended to bipartite begomovirus TGMV which is a mesophyll invasive virus (Morra & Petty, 2000) and partially releases the phloem specificity of TLCV (Rasheed et al., 2006) (chapter 4). *N. benthamiana* were co-inoculated with TGMV and DNA β construct. Interestingly DNA β was not found to spread systemically in more than two dozen plants co-inoculated with TGMV (data not shown). This is consistent with the observation that DNA β associated with AYVV is unable to produce systemic infection with SLCMV despite AYVV DNA β transreplicate with SLCMV (Saunders et al., 2002). Together these results suggest that satellite DNA β interact with phloem specific helper viruses to spread systemically and are localized in the phloem cells. A more comprehensive study is required to understand the association of this phloem specific begomovirus-DNA β disease complex to design control strategies for this increasing threat to global agriculture.

Chapter 8 - General discussion and concluding remarks

Geminiviruses are a large and diverse family of plant pathogens (Boulton, 2003; Moffat, 1999). Geminivirus epidemics have been attributed to their ability to undergo genetic recombination, their association with infectious satellite molecules, and their transmission by efficient insect vectors (Boulton, 2003; Polston & Anderson, 1999; Varma & Malathi, 2003). The majority of viruses of this family belong to the genus *Begomovirus*, which have enormous economic impact on global production of a wide range of plant species including vegetables, field and fibre crops (Boulton, 2003; Moffat, 1999). The Australian isolate of TLCV is a tomato-infecting geminivirus of major importance which has caused 80-100% crop damage in northern parts of Australia (Stonor et al., 2003) and there is a potential threat from the spread of this virus into the major tomato growing regions of Australia. Effective control of TLCV by transgenic resistance based on pathogen-derived resistance has not been successful (see section 1.5.2).

The mechanism of TLCV pathogenicity is not well understood. The work described in this thesis was undertaken with the objective of examining the location and functions of some of the gene products of TLCV at the cellular level. In situ hybridization procedures were developed to address this objective. These include strand specific probing, hybridization under denaturing and non-denaturing conditions, enzymatic removal of RNA transcripts before hybridization and tissue embedding using plastic resin (see chapter 3).

A distinct cytoplasmic localization pattern of TLCV ssDNA was observed which contrasts with the existing understanding of geminiviral intracellular localization (chapter 4). TLCV dsDNA was detected in the nucleus which agrees with previous conclusions that geminiviruses replicate in the nucleus. Consistent with earlier observations, both ss- and ds-DNA of the bipartite begomovirus TGMV were found in the nuclei. The new observation that TLCV is also located in the cytoplasm suggests that ssDNA is involved in cell-to-cell and long distance movement of this virus. This is supported by the observation that ssDNA is present in some of the xylem vessels.

Following TLCV replication in the nucleus, the next stage of infection is to spread to neighboring cells and to other tissues. A study elucidating movement mechanisms of TLCV is described in chapter 5. Mutants of TLCV with mutations in the *v1* and *c4* ORFs spread systemically, but less efficiently than wild-type virus. TLCV containing a mutation in the CP (*v2*) ORF was unable to infect plants systemically, but could replicate in the inoculated cells. To further investigate their role in viral movement, each of these proteins was fused with GFP and their intracellular localization was studied along with a host factor, SlUPTG1. TLCV CP accumulated in the nucleus, specifically in the nucleoli, and appeared to be functionally analogous to the NSP of bipartite begomoviruses. Both V1 and C4 targeted the PM and showed a specific association with Pd. The targeting of V1 and C4 to PM and/or Pd possibly occurred via the Golgi apparatus. Co-localization of V1 with ER was confirmed by rhodamine B-hexyl ester staining. V1 was also detected inside the nucleus which contrasts with the observation that V1 of TYLCV is found around, but not inside, the nucleus (Rojas et al., 2001). The sub-cellular localization of the tomato host protein, SlUPG1, which is a reversibly glycosylated peptide and interacts with V1 (Selth et al., 2006) was similar to the

localization pattern of V1 and supports the view this host factor could have a role in viral movement. Thus, three TLCV encoded proteins, CP, V1 and C4, and a host factor, SlUPTG1, are involved in viral movement.

Previous studies have shown that pathogen-derived transgenic resistance has not been successful against TLCV (see section 1.5.2) (Bian et al., 2006). In this thesis an attempt has been made to understand why this is so by determining the effect of *C4* transgene on TLCV replication (chapter 6). TLCV-derived *35S:C4* transgene was silenced upon TLCV infection not only in the phloem, but also in mesophyll cells where TLCV was not detectable. A high level of asymmetric methylation was found in the *35S:C4* transgene upon TLCV infection and this may have been responsible for the observed silencing. A high level of methylation of both symmetric and asymmetric cytosines was also found in TLCV RF DNA. However replication of TLCV continued in the silenced background. A model is proposed to explain the evasion of TLCV from host defence by synthesizing a population of unmethylated transcriptionally active DNA (Fig. 6.4). This could explain why pathogen-derived transgenic resistance has not been achieved against TLCV.

Satellite DNA β is becoming an emerging threat to global agriculture by its association with an increasing number of begomoviruses and because it enhances pathogenicity. The tissue tropism of CLCuMV associated DNA β was investigated. This DNA β was localized with TLCV in the phloem. The first satellite DNA was reported from Australia (Dry et al., 1997) but no DNA β-associated disease has appeared in Australia. Recently TYLCV was observed in large areas of Queensland, Australia (see section 1.5.2) suggesting that exclusion of such viruses by Australian quarantine will not be

effective over the long time. Since satellite DNA β molecules can interact with different phloem-specific monopartite begomoviruses they provide an additional risk through their ability to enhance the severity of existing and newly introduced monopartite begomoviruses.

This thesis describes three novel features of TLCV infection. These are (i) the cytoplasmic localization of TLCV ssDNA, (ii) a combined role for TLCV encoded proteins and a host factor in virus movement, and (iii) silencing of a TLCV-derived transgene and escape of TLCV from the host silencing mechanism. Figure 8.1 provides a model from the replication of TLCV based on the results described in this thesis. TLCV replicates in the nucleus through dsDNA (chapter 4) possibly by RCR and RDR. dsDNA could generate ssDNA which may either bind with CP to yield nucleoprotein complexes or progeny virions, or re-enter the replication cycle. RNA transcripts could be produced through RF DNA to enter the cytoplasm and lead to the synthesis of proteins which regulate various functions including viral replication, movement and host defence. It has been proposed that RNA transcripts of opposite polarity produced during transcription of geminiviruses could generate dsRNA (Chellappan et al., 2004b). This dsRNA possibly results in the production of siRNA through a host defence mechanism triggered by TLCV. TLCV-specific siRNA was observed upon TLCV infection, and this may have a role in the methylation of TLCV RF DNA (chapter 6). However TLCV synthesized a population of transcriptionally active unmethylated DNA through RDR and this escapes from silencing (chapter 6). Two other proteins, Rep (C1) and REn (C3), have a direct role in viral replication. A plant homologue of the retinoblastoma protein (RBR) binding domain has been identified in the TGMV Rep and supports the notion that Rep of begomoviruses plays an important role in infection

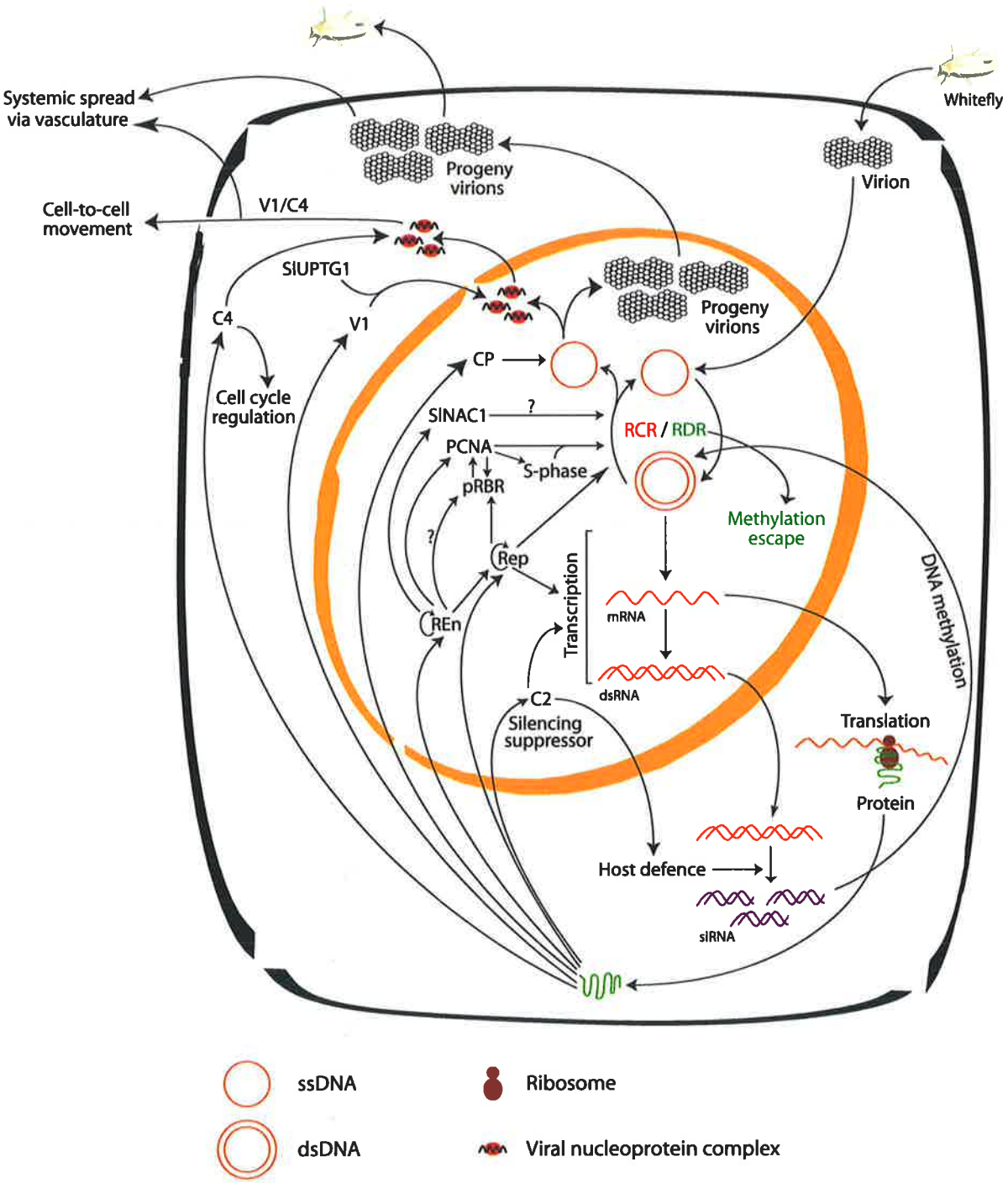


Figure 8.1. A model depicting TLCV infection with emphasis on replication, transcription and translation. Roles of different viral encoded proteins are indicated. RCR = rolling circle replication; RDR = recombination dependent replication; Rep = replication protein; REn = replication enhancer protein; CP = coat protein; SlNAC1 = REn host interactor protein; SlUPTG1 = V1 host interactor protein; PCNA = proliferating cell nuclear antigen; pRBR = a plant homolog of retinoblastoma related protein.

(Kong et al., 2000). It has been shown that REn binds to proliferating cell nuclear antigen (PCNA) and RBR, consistent with a role in replication and cell cycle regulation (Castillo et al., 2003; Settlage et al., 2001). In addition, TLCV-REn interacts with SlNAC1, a member of the plant NAC domain from tomato, and enhances TLCV replication (Selth et al., 2005). The expression of *SlNAC* was upregulated in TLCV infected cells (chapter 3). It is important to explore the mechanism(s) of these interactions in order to understand geminivirus replication, infection and pathogenesis. The *C4* coding region of TLCV is located entirely within the Rep coding region but in a different ORF (Fig 1.4). C4 is involved in symptom development (Krake et al., 1998), and may have a role in cell cycle regulation. This is supported by the observation that transgenic tobacco plants carrying the *C4* transgene exhibit hyperplasia (chapter 7). C4 may also have a role in cell-to-cell movement together with V1 of TLCV (chapter 5). V1 interacts with SlUPTG1 and both are associated with PM, Pd, ER and also localized inside the nucleus (chapter 5). Determining the precise role of SlUPTG1 in the virus movement pathway requires additional work. Newly synthesized virions and viral nucleoprotein complexes are thought to exit the nucleus and are transported through the cytoplasm to Pd for their active passage to the next cell. The efficient cell-to-cell transportation of the viral genome may require a cooperative role of V1 and C4.

The results of this study provide an insight into TLCV infection. Moreover, this study sets the direction for future research. For example (i) the significance of TLCV ssDNA accumulation in cytoplasm, (ii) the involvement of secretory pathway and endomembrane system in intracellular targeting of TLCV encoded movement proteins, (iii) the precise mechanism(s) of V1/SlUPTG1 in viral movement, (iv) the role of SlNAC1 in viral replication, and (v) methylation escape of TLCV through RDR. Such

information is expected to provide possibilities to develop a durable resistance against TLCV.

Reference list

Aaziz, R., Dinant, S. & Epel, B. L. (2001). Plasmodesmata and plant cytoskeleton. *Trends Plant Sci.* **6**, 326-330.

Abraham, T. W. (2001). Preparation of nonradioactive probes for in situ hybridization. *Methods* **23**, 297-302.

Alberter, B., Rezaian, M. A. & Jeske, H. (2005). Replicative intermediates of Tomato leaf curl virus and its satellite DNAs. *Virology* **331**, 441-448.

Al-Kaff, N. S., Covey, S. N., Kreike, M. M., Page, A. M., Pinder, R. & Dale, P. J. (1998). Transcriptional and posttranscriptional plant gene silencing in response to a pathogen. *Science* **279**, 2113-2115.

Anandalakshmi, R., Pruss, G. J., Ge, X., Marathe, R., Mallory, A. C., Smith, T. H. & Vance, V. B. (1998). A viral suppressor of gene silencing in plants. *Proc. Natl. Acad. Sci. USA* **95**, 13079-13084.

Angerer, L. M., Cox, K. H. & Angerer, R. C. (1987). Demonstration of tissue-specific gene expression by in situ hybridization. *Methods Enzymol.* **152**, 649-661.

Aoyama, T. & Chua, N. H. (1997). A glucocorticoid-mediated transcriptional induction system in transgenic plants. *Plant J.* **11**, 605-612.

Arguello-Astorga, G. R., Guevara-Gonzalez, R. G., Herrera-Estrella, L. R. & Rivera-Bustamante, R. F. (1994). Geminivirus replication origins have a group-specific organization of iterative elements: a model for replication. *Virology* **203**, 90-100.

Atkinson, R. G., Bieleski, L. R. F., Gleave, A. P., Janssen, B. J. & Morris, B. A. M. (1998). Post-transcriptional silencing of chalcone synthase in petunia using a geminivirus-based episomal vector. *Plant J.* **15**, 593-604.

Baas, P. D. (1987). Mutational analysis of the bacteriophage phi X174 replication origin. *J. Mol. Biol.* **198**, 51-61.

Baluska, F., Samaj, J., Napier, R. & Volkmann, D. (1999). Maize calreticulin localizes preferentially to plasmodesmata in root apex. *Plant J.* **19**, 481-488.

Barker, H., McGeachy, K. D., Ryabov, E. V., Commandeur, U., Mayo, M. A. & Taliansky, M. (2001). Evidence for RNA-mediated defence effects on the accumulation of Potato leafroll virus. *J. Gen. Virol.* **82**, 3099-3106.

Bass, H. W., Nagar, S., Hanley-Bowdoin, L. & Robertson, D. (2000). Chromosome condensation induced by geminivirus infection of mature plant cells. *J. Cell Sci.* **113**, 1149-1160.

Bayer, E., Thomas, C. L. & Maule, A. J. (2004). Plasmodesmata in Arabidopsis thaliana suspension cells. *Protoplasma* **223**, 93-102.

Behjatnia, S. A. A. (1997). Characterization of DNA replication of Tomato Leaf Curl Geminivirus. In *Department of Crop Protection*, pp. 102-122. Adelaide, Australia: The University of Adelaide.

Behjatnia, S. A. A., Dry, I. B. & Ali Rezaian, M. (1998). Identification of the replication-associated protein binding domain within the intergenic region of tomato leaf curl geminivirus. *Nucleic Acids Res.* **26**, 925-931.

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

Bernstein, E., Caudy, A. A., Hammond, S. M. & Hannon, G. J. (2001). Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature* **409**, 363-366.

Bian, X. Y., Rasheed, M. S., Seemanpillai, M. J. & Ali Rezaian, M. (2006). Analysis of silencing escape of tomato leaf curl virus: an evaluation of the role of DNA methylation. *Mol. Plant-Microbe Interact.* **19**, 614-624.

Blackman, L. M., Boevink, P., Cruz, S. S., Palukaitis, P. & Oparka, K. J. (1998). The movement protein of cucumber mosaic virus traffics into sieve elements in minor veins of Nicotiana Clevelandii. *Plant Cell* **10**, 525-538.

Boisnard-Lorig, C., Colon-Carmona, A., Bauch, M., Hodge, S., Doerner, P., Bancharel, E., Dumas, C., Haseloff, J. & Berger, F. (2001). Dynamic analyses of the expression of the HISTONE::YFP fusion protein in arabidopsis show that syncytial endosperm is divided in mitotic domains. *Plant Cell* **13**, 495-509.

Boulton, M. I. (2002). Functions and interactions of mastrevirus gene products. *Physiol. Mol. Plant Pathol.* **60**, 243-255.

Boulton, M. I. (2003). Geminiviruses: major threats to world agriculture. *Ann. Appl. Biol.* **142**, 143-143.

Boulton, M. I., Pallaghy, C. K., Chatani, M., MacFarlane, S. & Davies, J. W. (1993). Replication of maize streak virus mutants in maize protoplasts: evidence for a movement protein. *Virology* **192**, 85-93.

Boulton, M. I., Steinkellner, H., Donson, J., Markham, P. G., King, D. I. & Davies, J. W. (1989). Mutational analysis of the virion-sense genes of maize streak virus. *J. Gen. Virol.* **70**, 2309-2323.

Boyce, C. K., Zwieniecki, M. A., Cody, G. D., Jacobsen, C., Wirick, S., Knoll, A. H. & Holbrook, N. M. (2004). Evolution of xylem lignification and hydrogel transport regulation. *Proc. Natl. Acad. Sci. USA* **101**, 17555-17558.

Boyko, V., Ferralli, J., Ashby, J., Schellenbaum, P. & Heinlein, M. (2000). Function of microtubules in intercellular transport of plant virus RNA. *Nat. Cell Biol.* **2**, 826-832.

Briddon, R. W., Bull, S. E., Amin, I., Idris, A. M., Mansoor, S., Bedford, I. D., Dhawan, P., Rishi, N., Siwatch, S. S., Abdel-Salam, A. M., Brown, J. K., Zafar, Y. & Markham, P. G. (2003). Diversity of DNA beta, a satellite molecule associated with some monopartite begomoviruses. *Virology* **312**, 106-121.

Briddon, R. W., Mansoor, S., Bedford, I. D., Pinner, M. S. & Markham, P. G. (2000). Clones of cotton leaf curl geminivirus induce symptoms atypical of cotton leaf curl disease. *Virus Genes* **20**, 19-26.

Briddon, R. W., Mansoor, S., Bedford, I. D., Pinner, M. S., Saunders, K., Stanley, J., Zafar, Y., Malik, K. A. & Markham, P. G. (2001). Identification of DNA components required for induction of cotton leaf curl disease. *Virology* **285**, 234-243.

Briddon, R. W. & Markham, P. G. (2001). Complementation of bipartite begomovirus movement functions by topocuviruses and curtoviruses. *Arch. Virol.* **146**, 1811-1819.

Briddon, R. W., Pinner, M. S., Stanley, J. & Markham, P. G. (1990). Geminivirus coat protein replacement alters insect specificity. *Virology* **177**, 85-94.

Briddon, R. W. & Stanley, J. (2006). Subviral agents associated with plant single-stranded DNA viruses. *Virology* **344**, 198-210.

Brigneti, G., Voinnet, O., Li, W. X., Ji, L. H., Ding, S. W. & Baulcombe, D. C. (1998). Viral pathogenicity determinants are suppressors of transgene silencing in Nicotiana benthamiana. *EMBO J.* **17**, 6739-6746.

Brough, C. L., Gardiner, W. E., Inamdar, N. M., Zhang, X. Y., Ehrlich, M. & Bisaro, D. M. (1992). DNA methylation inhibits propagation of tomato golden mosaic virus DNA in transfected protoplasts. *Plant Mol. Biol.* **18**, 703-712.

Brown, J. K. (2000). Molecular markers for the identification and global tracking of whitefly vector-Begomovirus complexes. *Virus Res.* **71**, 233-260.

Brunetti, A., Tavazza, M., Noris, E., Tavazza, R., Caciagli, P., Ancora, G., Crespi, S. & Accotto, G. P. (1997). High expression of truncated viral rep protein confers resistance to tomato yellow leaf curl virus in transgenic tomato plants. *Mol. Plant-Microbe Interact.* **10**, 571-579.

Brunetti, A., Tavazza, R., Noris, E., Lucioli, A., Accotto, G. P. & Tavazza, M. (2001). Transgenically expressed T-Rep of tomato yellow leaf curl Sardinia virus acts as a trans-dominant-negative mutant, inhibiting viral transcription and replication. *J. Virol.* **75**, 10573-10581.

Burton, R. A., Gibeaut, D. M., Bacic, A., Findlay, K., Roberts, K., Hamilton, A., Baulcombe, D. C. & Fincher, G. B. (2000). Virus-induced silencing of a plant cellulose synthase gene. *Plant Cell* **12**, 691-706.

Butler, K., Zorn, A. M. & Gurdon, J. B. (2001). Nonradioactive in situ hybridization to xenopus tissue sections. *Methods* **23**, 303-312.

Cao, X., Aufsatz, W., Zilberman, D., Mette, M. F., Huang, M. S., Matzke, M. & Jacobsen, S. E. (2003). Role of the DRM and CMT3 methyltransferases in RNA-directed DNA methylation. *Curr. Biol.* **13**, 2212-2217.

Carrington, J. C., Kasschau, K. D., Mahajan, S. K. & Schaad, M. C. (1996). Cell-to-Cell and Long-Distance Transport of Viruses in Plants. *Plant Cell* **8**, 1669-1681.

Carter, C. J., Bednarek, S. Y. & Raikhel, N. V. (2004). Membrane trafficking in plants: new discoveries and approaches. *Curr. Opin. Plant Biol.* **7**, 701-707.

Casey, J. & Davidson, N. (1977). Rates of formation and thermal stabilities of RNA:DNA and DNA:DNA duplexes at high concentrations of formamide. *Nucleic Acids Res.* **4**, 1539-1552.

Castillo, A. G., Collinet, D., Deret, S., Kashoggi, A. & Bejarano, E. R. (2003). Dual interaction of plant PCNA with geminivirus replication accessory protein (Ren) and viral replication protein (Rep). *Virology* **312**, 381-394.

Cerutti, H. (2003). RNA interference: traveling in the cell and gaining functions? *Trends Genet.* **19**, 39-46.

Chellappan, P., Masona, M. V., Vanitharani, R., Taylor, N. J. & Fauquet, C. M. (2004a). Broad spectrum resistance to ssDNA viruses associated with transgene-induced gene silencing in cassava. *Plant Mol. Biol.* **56**, 601-611.

Chellappan, P., Vanitharani, R. & Fauquet, C. M. (2004b). Short interfering RNA accumulation correlates with host recovery in DNA virus-infected hosts, and gene silencing targets specific viral sequences. *J. Virol.* **78**, 7465-7477.

Choi, I. R. & Stenger, D. C. (1995). Strain-specific determinants of beet curly top geminivirus DNA replication. *Virology* **206**, 904-912.

Choi, I. R. & Stenger, D. C. (1996). The strain-specific cis-acting element of beet curly top geminivirus DNA replication maps to the directly repeated motif of the Ori. *Virology* **226**, 122-126.

Citovsky, V., Knorr, D., Schuster, G. & Zambryski, P. (1990). The P30 movement protein of tobacco mosaic virus is a single-strand nucleic acid binding protein. *Cell* **60**, 637-647.

Clark, S. J. & Frommer, M. (1997). Bisulphite genomic sequencing of methylated cytosines. In *Laboratory Methods for the Detection of Mutations and*

Polymorphisms in DNA, pp. 151-161. Edited by G. R. Taylor. Boca Raton: CRC Press.

Covey, S. N. & Al-Kaff, N. S. (2000). Plant DNA viruses and gene silencing. *Plant Mol. Biol.* **43**, 307-322.

Cui, X., Li, G., Wang, D., Hu, D. & Zhou, X. (2005). A Begomovirus DNAbeta-encoded protein binds DNA, functions as a suppressor of RNA silencing, and targets the cell nucleus. *J. Virol.* **79**, 10764-10775.

Cui, X., Tao, X., Xie, Y., Fauquet, C. M. & Zhou, X. (2004). A DNAbeta associated with Tomato yellow leaf curl China virus is required for symptom induction. *J. Virol.* **78**, 13966-13974.

Czosnek, H., Ber, R., Navot, N., Antignus, Y., Cohen, S. & Zamir, D. (1989). Tomato yellow leaf curl virus DNA forms in the viral capsid, in infected plants and in the insect vector. *J. Phytopathol.* **125**, 47-54.

Day, A. G., Bejarano, E. R., Buck, K. W., Burrell, M. & Lichtenstein, C. P. (1991). Expression of an antisense viral gene in transgenic tobacco confers resistance to the DNA virus tomato golden mosaic virus. *Proc. Natl. Acad. Sci. USA* **88**, 6721-6725.

de Almeida Engler, J., De Groodt, R., Van Montagu, M. & Engler, G. (2001). In situ hybridization to mRNA of Arabidopsis tissue sections. *Methods* **23**, 325-334.

De Barro, P. J. & Hart, P. J. (2000). Mating interactions between two biotypes of the whitefly, Bemisia tabaci (Hemiptera: Aleyrodidae) in Australia. *Bull Entomol. Res.* **90**, 103-112.

Desbiez, C., David, C., Mettouchi, A., Laufs, J. & Gronenborn, B. (1995). Rep protein of tomato yellow leaf curl geminivirus has an ATPase activity required for viral DNA replication. *Proc. Natl. Acad. Sci. USA* **92**, 5640-5644.

Dicenta, F., Martinez-Gomez, P., Rubio, M. & Audergon, J. M. (2003). Localisation and movement of Plum pox virus in apricot stem tissues. *Anal. App. Biol.* **142**, 99-105.

Dieguez, M. J., Bellotto, M., Afsar, K., Mittelsten Scheid, O. & Paszkowski, J. (1997). Methylation of cytosines in nonconventional methylation acceptor sites can contribute to reduced gene expression. *Mol. Gen. Genet.* **253**, 581-588.

Ding, B., Itaya, A. & Woo, Y. M. (1999a). Plasmodesmata and cell-to-cell communication in plants. *Int. Rev. Cytol.* **190**, 251-316.

Ding, X. S., Flasinski, S. & Nelson, R. S. (1999b). Infection of barley by brome mosaic virus is restricted predominantly to cells in and associated with veins through a temperature-dependent mechanism. *Mol. Plant-Microbe Interact.* **12**, 615-623.

Ding, X. S., Shintaku, M. H., Arnold, S. A. & Nelson, R. S. (1995). Accumulation of mild and severe strains of tobacco mosaic virus in minor veins of tobacco. *Mol. Plant-Microbe Interact.* **8**, 32-40.

Dong, X., van Wezel, R., Stanley, J. & Hong, Y. (2003). Functional characterization of the nuclear localization signal for a suppressor of posttranscriptional gene silencing. *J. Virol.* **77**, 7026-7033.

Dry, I., Krake, L., Mullineaux, P. & Rezaian, A. (2000). Regulation of tomato leaf curl viral gene expression in host tissues. *Mol. Plant-Microbe Interact.* **13**, 529-537.

Dry, I. B., Krake, L. R., Rigden, J. E. & Rezaian, M. A. (1997). A novel subviral agent associated with a geminivirus: the first report of a DNA satellite. *Proc. Natl. Acad. Sci. USA* **94**, 7088-7093.

Dry, I. B., Rigden, J. E., Krake, L. R., Mullineaux, P. M. & Rezaian, M. A. (1993). Nucleotide sequence and genome organization of tomato leaf curl geminivirus. *J. Gen. Virol.* **74**, 147-151.

Duan, Y. o. i., Powell, C. A., Purcifull, D. E., Broglio, P., Hiebert, E. & Duan, Y. P. (1997). Phenotypic variation in transgenic tobacco expressing mutated geminivirus movement/pathogenicity (BC1) proteins. *Mol. Plant-Microbe Interact.* **10**, 1065-1074.

Eagle, P. A. & Hanley-Bowdoin, L. (1997). cis Elements that contribute to Geminivirus transcriptional regulation and the efficiency of DNA replication. *J. Virology* **71**, 6947-6955.

Eagle, P. A., Orozco, B. M. & Hanley-Bowdoin, L. (1994). A DNA sequence required for geminivirus replication also mediates transcriptional regulation. *Plant Cell* **6**, 1157-1170.

Egelkrout, E. M., Robertson, D. & Hanley-bowdoin, L. (2001). Proliferating cell nuclear antigen transcription is repressed through an E2F consensus element and activated by geminivirus infection in mature leaves. *Plant Cell* **13**, 1437- 1452.

Elmer, J. S., Brand, L., Sunter, G., Gardiner, W. E., Bisaro, D. M. & Rogers, S. G. (1988). Genetic analysis of the tomato golden mosaic virus II. The product of the AL1 coding sequence is required for replication. *Nucleic Acids Res.* **16**, 7043-7060.

Ermak, G., Paszkowski, U., Wohlmuth, M., Scheid, O. M. & Paszkowski, J. (1993). Cytosine methylation inhibits replication of African cassava mosaic virus by two distinct mechanisms. *Nucleic Acids Res.* **21**, 3445-3450.

Fauquet, C. M., Bisaro, D. M., Briddon, R. W., Brown, J. K., Harrison, B. D., Rybicki, E. P., Stenger, D. C. & Stanley, J. (2003). Revision of taxonomic criteria for species demarcation in the family Geminiviridae, and an updated list of begomovirus species. *Arch. Virol.* **148**, 405-421.

Fofana, I. B., Sangare, A., Collier, R., Taylor, C. & Fauquet, C. M. (2004). A geminivirus-induced gene silencing system for gene function validation in cassava. *Plant Mol. Biol.* **56**, 613-624.

Fontes, E. P. B., Eagle, P. A., Sipe, P. S., Luckow, V. A. & Hanley-Bowdoin, L. (1994a). Interaction between a geminivirus replication protein and origin DNA is essential for viral replication. *J. Biol. Chem.* **269**, 8459-8465.

Fontes, E. P. B., Gladfelter, H. J., Schaffer, R. L., Petty, I. T. D. & Hanley-Bowdoin, L. (1994b). Geminivirus replication origins have a modular organization. *Plant Cell* **6**, 405-416.

Fontes, E. P. B., Luckow, V. A. & Hanley-Bowdoin, L. (1992). A geminivirus replication protein is a sequence-specific DNA binding protein. *Plant Cell* **4**, 597-608.

Frischmuth, T. & Stanley, J. (1992). Characterization of beet curly top virus subgenomic DNA localizes sequences required for replication. *Virology* **189**, 808-811.

Fujiwara, T., Giesman-Cookmeyer, D., Ding, B., Lommel, S. A. & Lucas, W. J. (1993). Cell-to-Cell Trafficking of Macromolecules through Plasmodesmata Potentiated by the Red Clover Necrotic Mosaic Virus Movement Protein. *Plant Cell* **5**, 1783-1794.

Fujiwara, T., Oda, K., Yokota, S., Takatsuki, A. & Ikehara, Y. (1988). Brefeldin A causes disassembly of the Golgi complex and accumulation of secretory proteins in the endoplasmic reticulum. *J. Biol. Chem.* **263**, 18545-18552.

Fukuda, H. (1997). Tracheary Element Differentiation. *Plant Cell* **9**, 1147-1156.

Fulnecek, R. & Matyasek (1998). Mapping of 5-methylcytosine residues in Nicotiana tabacum 5S rRNA genes by genomic sequencing. *Mol. Gen. Genet.* **259**, 133-141.

Gafni, Y. & Bernard, L. E. (2002). The role of host and viral proteins in intra- and inter-cellular trafficking of geminiviruses. *Physiol. Mol. Plant Pathol.* **60**, 231-241.

Gall, J. G. & Pardue, M. L. (1969). Formation and detection of RNA-DNA hybrid molecules in cytological preparations. *Proc. Natl. Acad. Sci. USA* **63**, 378-383.

Gilbertson, R. L. & Lucas, W. J. (1996). How do viruses traffic on the 'vascular highway'? *Trends Plant Sci.* **1**, 260-268.

Gillespie, T., Boevink, P., Haupt, S., Roberts, A. G., Toth, R., Valentine, T., Chapman, S. & Oparka, K. J. (2002). Functional analysis of a DNA-shuffled movement protein reveals that microtubules are dispensable for the cell-to-cell movement of tobacco mosaic virus. *Plant Cell* **14**, 1207-1222.

Gladfelter, H. J., Eagle, P. A., Fontes, E. P. B., Batts, L. A. & Hanley-Bowdoin, L. (1997). Two domains of the AL1 protein mediate geminivirus origin recognition. *Virology* **239**, 186-197.

Gleave, A. P. (1992). A versatile binary vector system with a T-DNA organisational structure conducive to efficient integration of cloned DNA into the plant genome. *Plant Mol. Biol.* **20**, 1203-1207.

Gorbalenya, A. E. & Koonin, E. V. (1989). Viral proteins containing the purine NTP-binding sequence pattern. *Nucleic Acids Res.* **17**, 8413-8440.

Gorshkova, E. N., Erokhina, T. N., Stroganova, T. A., Yelina, N. E., Zamyatnin, A. A., Jr., Kalinina, N. O., Schiemann, J., Solovyev, A. G. & Morozov, S. Y. (2003). Immunodetection and fluorescent microscopy of transgenically expressed hordeivirus TGBp3 movement protein reveals its association with endoplasmic reticulum elements in close proximity to plasmodesmata. *J. Gen. Virol.* **84**, 985-994.

Grimsley, N., Hohn, T., Davies, J. W. & Hohn, B. (1987). Agrobacterium-mediated delivery of infectious maize streak virus into maize plants. *Nature* **325**, 177-179.

Gruenbaum, Y., Naveh-Many, T., Cedar, H. & Razin, A. (1981). Sequence specificity of methylation in higher plant DNA. *Nature* **292**, 860-862.

Grunau, C., Hindermann, W. & Rosenthal, A. (2000). Large-scale methylation analysis of human genomic DNA reveals tissue-specific differences between the methylation profiles of genes and pseudogenes. *Human Mol. Gen.* **9**, 2651-2663.

Guan, C. & Zhou, X. (2006). Phloem specific promoter from a satellite associated with a DNA virus. *Virus Res.* **115**, 150-157.

Gubler, F. (1989). Immunofluorescence localisation of microtubules in plant root tips embedded in butyl-methyl methacrylate. *Cell Biol. Int. Rep.* **13**, 137-145.

Gunning, R. V., Bryne, F. J., Conde, B. D., Connelly, M. I., Hergstrom, K. & Devonshire, A. L. (1995). First report of B-Biotype *Bemisia tabaci* (Hemiptera: Aleyrodidae) in Australia. *J. Australian Entomol. Soc.* **34**, 116.

Gutierrez, C. (1999). Geminivirus DNA replication. *Cell. Mol. Life Sci.* **56**, 313-329.

Gutierrez, C. (2000). DNA replication and cell cycle in plants: learning from geminiviruses. *EMBO J.* **19**, 792-799.

Haley, A., Zhan, X., Richardson, K., Head, K. & Morris, B. (1992). Regulation of the activities of African cassava mosaic virus promoters by the AC1, AC2, and AC3 gene products. *Virology* **188**, 905-909.

Hamilton, A. J. & Baulcombe, D. C. (1999). A species of small antisense RNA in posttranscriptional gene silencing in plants. *Science* **286**, 950-952.

Hammond, S. M., Bernstein, E., Beach, D. & Hannon, G. J. (2000). An RNA-directed nuclease mediates post-transcriptional gene silencing in *Drosophila* cells. *Nature* **404**, 293-296.

Hanley-Bowdoin, L., Settlage, S. B., Orozco, B. M., Nagar, S. & Robertson, D. (1999). Geminiviruses: models for plant DNA replication, transcription, and cell cycle regulation. *Crit. Rev. Plant Sci.* **18**, 71-106.

Harrison, B. D. & Robinson, D. J. (2002). Green shoots of geminivirology. *Physiol. Mol. Plant Pathol.* **60**, 215-218.

Hauptmann, G. & Gerster, T. (1994). Two-color whole-mount in situ hybridization to vertebrate and Drosophila embryos. *Trends Genet* **10**, 266.

Hayes, R. J., Macdonald, H., Coutts, R. H. A. & Buck, K. W. (1988). Agroinfection of Triticum aestivum with cloned DNA of wheat dwarf virus. *J. Gen. Virol.* **69**, 891-896.

Hecht, K. (1912). Studien über den Vorgang der Plasmolyse. *Beitr. Biol. Pflanz.* **11**, 133-145.

Hehnle, S., Wege, C. & Jeske, H. (2004). Interaction of DNA with the movement proteins of geminiviruses revisited. *J. Virol.* **78**, 7698-7706.

Heinlein, M., Padgett, H. S., Gens, J. S., Pickard, B. G., Casper, S. J., Epel, B. L. & Beachy, R. N. (1998). Changing patterns of localization of the tobacco mosaic virus movement protein and replicase to the endoplasmic reticulum and microtubules during infection. *Plant Cell* **10**, 1107-1120.

Heyraud, F., Matzeit, V., Kammann, M., Schaefer, S., Schell, J. & Gronenborn, B. (1993). Identification of the initiation sequence for viral-strand DNA synthesis of wheat dwarf virus. *EMBO J.* **12**, 4445-4452.

Holliday, R. & Pugh, J. E. (1975). DNA modification mechanisms and gene activity during development. *Science* **187**, 226-232.

Holmes, D. S. & Quigley, M. (1981). A rapid boiling method for the preparation of bacterial plasmids. *Anal. Biochem.* **114**, 193-197.

Hong, Y., Saunders, K., Hartley, M. R. & Stanley, J. (1996). Resistance to geminivirus infection by virus-induced expression of dianthin in transgenic plants. *Virology* **220**, 119-127.

Hong, Y. & Stanley, J. (1995). Regulation of African cassava mosaic virus complementary-sense gene expression by N-terminal sequences of the replication-associated protein AC1.PG - 2415-22. *J. Gen. Virol.* **76**, 2415-2422.

Hormuzdi, S. G. & Bisaro, D. M. (1995). Genetic analysis of beet curly top virus: examination of the roles of L2 and L3 genes in viral pathogenesis. *Virology* **206**, 1044-1054.

Horns, T. & Jeske, H. (1991). Localization of abutilon mosaic virus (AbMV) DNA within leaf tissue by in situ hybridization. *Virology* **181**, 580-588.

Hou, Y. u. i., Sanders, R., Ursin, V. M., Gilbertson, R. L. & Hou, Y. M. (2000). Transgenic plants expressing geminivirus movement proteins: abnormal phenotypes and delayed infection by Tomato mottle virus in transgenic tomatoes expressing the Bean dwarf mosaic virus BV1 or BC1 proteins. *Mol. Plant-Microbe Interact.* **13**, 297-308.

Hull, R. (2002). Matthews' Plant Virology, 4th. edn. London: Academic Press.

Ingham, D. J., Pascal, E. & Lazarowitz, S. G. (1995). Both bipartite geminivirus movement proteins define viral host range, but only BL1 determines viral pathogenicity. *Virology* **207**, 191-204.

Jaenicke, R. (1991). Protein folding: local structures, domains, subunits, and assemblies. *Biochemistry* **30**, 3147-3161.

Jeske, H., Lutgemeier, M. & Preiss, W. (2001). DNA forms indicate rolling circle and recombination-dependent replication of Abutilon mosaic virus. *EMBO J* **20**, 6158-6167.

John, H. A., Birnstiel, M. L. & Jones, K. W. (1969). RNA-DNA hybrids at the cytological level. *Nature* **223**, 582-587.

Johnson, D. R., Bhatnagar, R. S., Knoll, L. J. & Gordon, J. I. (1994). Genetic and biochemical studies of protein N-myristoylation. *Annu. Rev. Biochem.* **63**, 869-914.

Jones, L., Hamilton, A. J., Voinnet, O., Thomas, C. L., Maule, A. J. & Baulcombe, D. C. (1999). RNA-DNA interactions and DNA methylation in post-transcriptional gene silencing. *Plant Cell* **11**, 2291-2301.

Jones, L., Ratcliff, F. & Baulcombe, D. C. (2001). RNA-directed transcriptional gene silencing in plants can be inherited independently of the RNA trigger and requires Met1 for maintenance. *Curr. Biol.* **11**, 747-757.

Jones, M. G. K. (1976). The origin and development of plasmodesmata. In *Intercellular Communication in Plants:Studies on Plasmodesmata*, pp. 81-105. Edited by B. E. S. Gunning & A. W. Robards. Heidelberg, Germany: Springer-Verlag.

Jose, J. & Usha, R. (2003). Bhendi yellow vein mosaic disease in India is caused by association of a DNA Beta satellite with a begomovirus. *Virology* **305**, 310-317.

Jowett, T. (2001). Double in situ hybridization techniques in zebrafish. *Methods* **23**, 345-358.

Jupin, I., Kouchkovsky, F. d., Jouanneau, F., Gronenborn, B. & De, K. o. (1994). Movement of tomato yellow leaf curl geminivirus (TYLCV): involvement of the protein encoded by ORF C4. *Virology* **204**, 82-90.

Kassanis, B. (1962). Properties and behaviour of a virus depending for its multiplication on another. *J. Gen. Microbiol.* **27**, 477-488.

Kasteel, D. T., Perbal, M. C., Boyer, J. C., Wellink, J., Goldbach, R. W., Maule, A. J. & van Lent, J. W. (1996). The movement proteins of cowpea mosaic virus and cauliflower mosaic virus induce tubular structures in plant and insect cells. *J. Gen. Virol.* **77**, 2857-2864.

Kheyr-Pour, A., Bendahmane, M., Matzeit, V., Accotto, G. P., Crespi, S. & Gronenborn, B. (1991). Tomato yellow leaf curl virus from Sardinia is a whitefly-transmitted monopartite geminivirus. *Nucleic Acids Res.* **19**, 6763-6769.

Kim, K. S. & Lee, K. W. (1992). Geminivirus-induced macrotubules and their suggested role in cell-to-cell movement. *Phytopathology* **82**, 664-669.

Kim, K. S., Shock, T. L. & Goodman, R. M. (1978). Infection of Phaseolus vulgaris by bean golden mosaic virus: ultrastructural aspects. *Virology* **89**, 22-33.

Kjemtrup, S., Sampson, K. S., Peele, C. G., Nguyen, L. V., Conkling, M. A., Thompson, W. F. & Robertson, D. (1998). Gene silencing from plant DNA carried by a geminivirus. *Plant J.* **14**, 91-100.

Kong, L. J., Orozco, B. M., Roe, J. L., Nagar, S., Ou, S., Feiler, H. S., Durfee, T., Miller, A. B., Gruissem, W., Robertson, D. & Hanley-Bowdoin, L. (2000). A geminivirus replication protein interacts with the retinoblastoma protein through a novel domain to determine symptoms and tissue specificity of infection in plants. *EMBO J.* **19**, 3485-3495.

Kopito, R. R. (2000). Aggresomes, inclusion bodies and protein aggregation. *Trends Cell Biol.* **10**, 524-530.

Kornberg, A. & Baker, T. A. (1992). DNA Replication, 2ed edn. New York: W. H. Freeman and Company.

Kotlizky, G., Katz, A., van der Laak, J., Boyko, V., Lapidot, M., Beachy, R. N., Heinlein, M. & Epel, B. L. (2001). A dysfunctional movement protein of tobacco mosaic virus interferes with targeting of wild-type movement protein to microtubules. *Mol. Plant-Microbe Interact.* **14**, 895-904.

Krake, L. R., Rezaian, M. A. & Dry, I. B. (1998). Expression of the tomato leaf curl geminivirus C4 gene produces viruslike symptoms in transgenic plants. *Mol. Plant-Microbe Interact.* **11**, 413-417.

Kronenberger, J., Desprez, T., Höfte, H., Caboche, M. & Traas, J. (1993). A methacrylate embedding procedure developed for immunolocalization on plant tissues is also compatible with in situ hybridization. *Cell Biol. Int.* **17**, 1013-1022.

Labrie, C., Lee, B. H. & Mathews, M. B. (1995). Transcription factors RFX1/EF-C and ATF-1 associate with the adenovirus E1A-responsive element of the human proliferating cell nuclear antigen promoter. *Nucleic Acids Res.* **23**, 3732-3741.

Lang-Pauluzzi, I. (2000). The behaviour of the plasma membrane during plasmolysis: a study by UV microscopy. *J. Microscopy* **198**, 188-198.

Laporte, C., Vetter, G., Loudes, A. M., Robinson, D. G., Hillmer, S., Stussi-Garaud, C. & Ritzenthaler, C. (2003). Involvement of the secretory pathway and the cytoskeleton in intracellular targeting and tubule assembly of Grapevine fanleaf virus movement protein in tobacco BY-2 cells. *Plant Cell* **15**, 2058-2075.

Latham, J. R., Saunders, K., Pinner, M. S. & Stanley, J. (1997). Induction of plant cell division by beet curly top virus gene C4. *Plant J.* **11**, 1273-1283.

Laufs, J., Jupin, I., David, C., Schumacher, S., Heyraud-Nitschke, F. & Gronenborn, B. (1995a). Geminivirus replication: genetic and biochemical characterization of Rep protein function, a review. *Biochimie.* **77**, 765-773.

Laufs, J., Traut, W., Heyraud, F., Matzeit, V., Rogers, S. G., Schell, J. & Gronenborn, B. (1995b). In vitro cleavage and joining at the viral origin of replication by the replication initiator protein of tomato yellow leaf curl virus. *Proc. Natl. Acad. Sci. USA* **92**, 3879-3883.

Lazarowitz, S. G. (1992). Geminiviruses: genome structure and gene function. *Crit. Rev. Plant Sci.* **11**, 327-349.

Lazarowitz, S. G. (1999). Probing plant cell structure and function with viral movement proteins. *Curr. Opin. Plant Biol.* **2**, 332-338.

Lazarowitz, S. G. & Beachy, R. N. (1999). Viral movement proteins as probes for intracellular and intercellular trafficking in plants. *Plant Cell* **11**, 535-548.

Lazarowitz, S. G., Pinder, A. J., Damsteegt, V. D. & Rogers, S. G. (1989). Maize streak virus genes essential for systemic spread and symptom development. *EMBO J.* **8**, 1023-1032.

Lazarowitz, S. G., Wu, L. C., Rogers, S. G. & Elmer, J. S. (1992). Sequence-specific interaction with the viral AL1 protein identifies a geminivirus DNA replication origin. *Plant Cell* **4**, 799-809.

Lecoq, K., Belloc, I., Desgranges, C. & Daignan-Fornier, B. (2001). Role of adenosine kinase in Saccharomyces cerevisiae: identification of the ADO1 gene and study of the mutant phenotypes. *Yeast* **18**, 335-342.

Lee, W. S., Kao, C. C., Bryant, G. O., Liu, X. & Berk, A. J. (1991). Adenovirus E1A activation domain binds the basic repeat in the TATA box transcription factor. *Cell* **67**, 365-376.

Lewandowski, D. J. & Dawson, W. O. (1998). Deletion of internal sequences results in tobacco mosaic virus defective RNAs that accumulate to high levels without interfering with replication of the helper virus. *Virology* **251**, 427-437.

Lin, B., Behjatnia, S. A. A., Dry, I. B., Randles, J. W. & Rezaian, M. A. (2003). High affinity Rep-binding is not required for the replication of a Geminivirus DNA and its satellite. *Virology* **305**, 353-363.

Liu, H., Boulton, M. I., Oparka, K. J. & Davies, J. W. (2001). Interaction of the movement and coat proteins of Maize streak virus: implications for the transport of viral DNA. *J. Gen. Virol* **82**, 35-44.

Liu, H., Boulton, M. I., Thomas, C. L., Prior, D. A. M., Oparka, K. J. & Davies, J. W. (1999). Maize streak virus coat protein is karyophyllic and facilitates nuclear transport of viral DNA. *Mol. Plant-Microbe Interact.* **12**, 894-900.

Liu, L., Davies, J. W. & Stanley, J. (1998). Mutational analysis of bean yellow dwarf virus, a geminivirus of the genus Mastrevirus that is adapted to dicotyledonous plants. *J. Gen. Virol.* **79**, 2265-2274.

Lucas, W. J. & Gilbertson, R. L. (1994). Plasmodesmata in relation to viral movement within leaf tissues. *Annu. Rev. Phytopathol.* **32**, 387-411.

Lucioli, A., Noris, E., Brunetti, A., Tavazza, R., Ruzza, V., Castillo, A. G., Bejarano, E. R., Accotto, G. P. & Tavazza, M. (2003). Tomato yellow leaf curl Sardinia virus rep-derived resistance to homologous and heterologous geminiviruses occurs by different mechanisms and is overcome if virus-mediated transgene silencing is activated. *J. Virol.* **77**, 6785-6798.

MacDonald, H., Coutts, R. H. A. & Buck, K. W. (1988). Characterization of a subgenomic DNA isolated from Triticum aestivum plants infected with wheat dwarf virus. *J. Gen. Virol.* **69**, 1339-1344.

Mansoor, S., Briddon, R. W., Zafar, Y. & Stanley, J. (2003). Geminivirus disease complexes: an emerging threat. *Trends Plant Sci.* **8**, 128-134.

Mansoor, S., Khan, S. H., Bashir, A., Saeed, M., Zafar, Y., Malik, K. A., Briddon, R., Stanley, J. & Markham, P. G. (1999). Identification of a novel circular single-stranded DNA associated with cotton leaf curl disease in Pakistan. *Virology* **259**, 190-199.

Mansoor, S., Zafar, Y. & Briddon, R. W. (2006). Geminivirus disease complexes: the threat is spreading. *Trends Plant Sci.* **11**, 209-212.

Martinez, M. C., Jorgensen, J. E., Lawton, M. A., Lamb, C. J. & Doerner, P. W. (1992). Spatial pattern of cdc2 expression in relation to meristem activity and cell proliferation during plant development. *Proc. Natl. Acad. Sci. USA* **89**, 7360-7364.

Mathews, R. E. F. (1991). Plant virology, 3rd edn. San Diego, California: Academic Press Inc.

Matzk, A., Mantell, S. & Schiemann, J. (1996). Localization of persisting Agrobacteria in transgenic tobacco plants. *Mol. Plant-Microbe Interact.* **9**, 373-381.

Mauseth, J. D. (1988). Plant Anatomy. California, USA: Benjamin/Cummings Publication Coperation.

Mayo, M. A., Leibowitz, M. J., Palukaitis, P., Scholthof, k.-B. G., Simon, A. E., Stanley, J. & Taliansky, M. (2005). Satellites. In *Virus Taxonomy: VIIIth Report of the International Committee on Taxonomy of Viruses*, pp. 1163-1169. Edited by C. M. Fauquet, M. A. Mayo, J. Maniloff, U. Desselberger & L. A. Ball. London: Elsevier/Academic Press.

McFadden, G. I. (1989). In situ hybridisation in plants: From macroscopic to ultrastructural resolution. *Cell Biol. Int. Rep.* **13**, 3-21.

McFadden, G. I., Bonig, I., Cornish, E. C. & Clarke, A. E. (1988). A simple fixation and embedding method for use in hybridization histochemistry on plant tissues. *Histochem. J.* **20**, 575-586.

McLean, B. G., Hempel, F. D. & Zambryski, P. C. (1997). Plant intercellular communication via plasmodesmata. *Plant Cell* **9**, 1043-1054.

Mette, M. F., Aufsatz, W., van der Winden, J., Matzke, M. A. & Matzke, A. J. (2000). Transcriptional silencing and promoter methylation triggered by double-stranded RNA. *EMBO J.* **19**, 5194-5201.

Moffat, A. S. (1999). Geminiviruses emerge as serious crop threat. *Science* **286**, 1835.

Moreno, I. M., Thompson, J. R. & Garcia-Arenal, F. (2004). Analysis of the systemic colonization of cucumber plants by Cucumber green mottle mosaic virus. *J. Gen. Virol.* **85**, 749-759.

Morilla, G., Krenz, B., Jeske, H., Bejarano, E. R. & Wege, C. (2004). Tete a tete of tomato yellow leaf curl virus and tomato yellow leaf curl sardinia virus in single nuclei. *J. Virol.* **78**, 10715-10723.

Morra, M. R. & Petty, I. T. (2000). Tissue specificity of geminivirus infection is genetically determined. *Plant Cell* **12**, 2259-2270.

Morris-Krsinich, B. A., Mullineaux, P. M., Donson, J., Boulton, M. I., Markham, P. G., Short, M. N. & Davies, J. W. (1985). Bidirectional transcription of maize streak virus DNA and identification of the coat protein gene. *Nucleic Acids Res.* **13**, 7237-7256.

Mullineaux, P. M., Rigden, J. E., Dry, I. B., Krake, L. R. & Rezaian, M. A. (1993). Mapping of the polycistronic RNAs of tomato leaf curl geminivirus. *Virology* **193**, 414-423.

Nagar, S., Hanley-Bowdoin, L. & Robertson, D. (2002). Host DNA replication is induced by geminivirus infection of differentiated plant cells. *Plant Cell* **14**, 2995-3007.

Nagar, S., Pedersen, T. J., Carrick, K. M., Hanley-Bowdoin, L. & Robertson, D. (1995). A geminivirus induces expression of a host DNA synthesis protein in terminally differentiated plant cells. *Plant Cell* **7**, 705-719.

Nakhla, M. K. & Maxwell, D. P. (1998). Epidemiology and management of tomato yellow leaf curl disease. In *Plant Virus Disease Control*, pp. 565-583. Edited by A. Hadidi, R. K. Khetarpal & H. K. Koganezawa: APS Press.

Napoli, C., Lemieux, C. & Jorgensen, R. (1990). Introduction of a chalcone synthease gene into *Petunia* results in reversible co-suppression of homologous genes in trans. *Plant Cell* **2**, 279-289.

Nebenfuhr, A., Ritzenthaler, C. & Robinson, D. G. (2002). Brefeldin A: deciphering an enigmatic inhibitor of secretion. *Plant Physiol.* **130**, 1102-1108.

Nellen, W. & Lichtenstein, C. (1993). What makes an mRNA anti-sensitive? *Trends Biochem. Sci.* **18**, 419-423.

Nelson, R. S. & van Bel, A. J. E. (1998). The mystery of virus trafficking into, through and out of vascular tissue. *Prog. Botany* **59**, 476-533.

Noris, E., Accotto, G. P., Tavazza, R., Brunetti, A., Crespi, S. & Tavazza, M. (1996). Resistance to tomato yellow leaf curl geminivirus in Nicotiana benthamiana plants transformed with a truncated viral C1 gene. *Virology* **224**, 130-138.

Noris, E., Lucioli, A., Tavazza, R., Caciagli, P., Accotto, G. P. & Tavazza, M. (2004). Tomoto yellow leaf curl Sardinia virus can overcome transgene-mediated RNA silencing of two essential viral genes. *J. Gen. Virol.* **85**, 1745-1749.

Noris, E., Vaira, A. M., Caciagli, P., Masenga, V., Gronenborn, B. & Accotto, G. P. (1998). Amino acids in the capsid protein of tomato yellow leaf curl virus that are crucial for systemic infection, particle formation, and insect transmission. *J. Virol.* **72**, 10050-10057.

Noueiry, A. O., Lucas, W. J. & Gilbertson, R. L. (1994). Two proteins of a plant DNA virus coordinate nuclear and plasmodesmal transport. *Cell* **76**, 925-932.

Novick, R. P. (1998). Contrasting lifestyles of rolling-circle phages and plasmids. *Trends Biochem. Sci.* **23**, 434-438.

Opalka, N., Brugidou, C., Bonneau, C., Nicole, M., Beachy, R. N., Yeager, M. & Fauquet, C. (1998). Movement of rice yellow mottle virus between xylem cells through pit membranes. *Proc. Natl. Acad. Sci. USA* **95**, 3323-3328.

Oparka, K. J., Prior, D. A. M. & Crawford, J. W. (1994). Behaviour of plasma membrane, cortical ER and plasmodesmata during plasmolysis of onion epidermal cells. *Plant Cell Environ.* **17**, 163-171.

Orozco, B. M., Gladfelter, H. J., Settlage, S. B., Eagle, P. A., Gentry, R. N. & Hanley-Bowdoin, L. (1998). Multiple cis elements contribute to geminivirus origin function. *Virology* **242**, 346-356.

Orozco, B. M. & Hanley-Bowdoin, L. (1996). A DNA structure is required for geminivirus replication origin function. *J. Virology* **70**, 148-158.

Orozco, B. M. & Hanley-Bowdoin, L. (1998). Conserved sequence and structural motifs contribute to the DNA binding and cleavage activities of a geminivirus replication protein. *J. Biol. Chem.* **273**, 24448-24456.

Orozco, B. M., Miller, A. B., Settlage, S. B. & Hanley-Bowdoin, L. (1997). Functional domains of a geminivirus replication protein. *J. Biol. Chem.* **272**, 9840-9846.

Padgett, H. S., Epel, B. L., Kahn, T. W., Heinlein, M., Watanabe, Y. & Beachy, R. N. (1996). Distribution of tobamovirus movement protein in infected cells and implications for cell-to-cell spread of infection. *Plant J.* **10**, 1079-1088.

Padidam, M., Beachy, R. N. & Fauquet, C. M. (1995). Classification and identification of geminiviruses using sequence comparisons. *J. Gen. Virol.* **76**, 249-263.

Padidam, M., Beachy, R. N. & Fauquet, C. M. (1999). A phage single-stranded DNA (ssDNA) binding protein complements ssDNA accumulation of a geminivirus and interferes with viral movement. *J. Virol.* **73**, 1609-1616.

Palanichelvam, K., Kunik, T., Citovsky, V. & Gafni, Y. (1998). The capsid protein of tomato yellow leaf curl virus binds cooperatively to single-stranded DNA. *J. Gen. Virol.* **79**, 2829-2833.

Pascal, E., Goodlove, P. E., Wu, L. C. & Lazarowitz, S. G. (1993). Transgenic tobacco plants expressing the geminivirus BL1 protein exhibit symptoms of viral disease. *Plant Cell* **5**, 795-807.

Pascal, E., Sanderfoot, A. A., Ward, B. M., Medville, R., Turgeon, R. & Lazarowitz, S. G. (1994). The geminivirus BR1 movement protein binds single-stranded DNA and localizes to the cell nucleus. *Plant Cell* **6**, 995-1006.

Pedersen, T. J. & Hanley-Bowdoin, L. (1994). Molecular characterization of the AL3 protein encoded by a bipartite geminivirus. *Virology* **202**, 1070-1075.

Peele, C., Jordan, C. V., Muangsan, N., Turnage, M., Egelkrout, E., Eagle, P., Hanley-bowdoin, L. & Robertson, D. (2001). Silencing of a meristematic gene using geminivirus-derived vectors. *Plant J.* **27**, 357- 366.

Pelissier, T., Thalmeir, S., Kempe, D., Sanger, H. L. & Wassenegger, M. (1999). Heavy *de novo* methylation at symmetrical and non-symmetrical sites is a hallmark of RNA-directed DNA methylation. *Nucleic Acids Res.* **27**, 1625-1634.

Pena, R. N., Webster, J., Kwan, S., Korbel, J. & Whitelaw, B. A. (2004). Transgene methylation in mice reflects copy number but not expression level. *Mol. Biotechnol.* **26**, 215-220.

Pickford, A. S. & Cogoni, C. (2003). RNA-mediated gene silencing. *Cell Mol. Life Sci.* **60**, 871-882.

Pilartz, M. & Jeske, H. (1992). Abutilon mosaic geminivirus double-stranded DNA is packed into minichromosomes. *Virology* **189**, 800-802.

Polston, J. E. & Anderson, P. K. (1999). The emergence of whitefly-transmitted geminiviruses in tomato in the western hemisphere. *Manejo Integrado de Plagas*, 24-42.

Pont-Lezica, R. F., McNally, J. G. & Pickard, B. G. (1993). Wall-to-membrane linkers in onion epidermis: some hypotheses. *Plant Cell Environ.* **16**, 111-123.

Preiss, W. & Jeske, H. (2003). Multitasking in replication is common among geminiviruses. *J. Virol.* **77**, 2972-2980.

Pringle, C. R. (1999). Virus taxonomy at the XIth International Congress of Virology, Sydney, Australia, 1999. *Arch. Virol.* **144**, 2065-2070.

Qin, S., Ward, B. M., Lazarowitz, S. G. & Qin, S. W. (1998). The bipartite geminivirus coat protein aids BR1 function in viral movement by affecting the accumulation of viral single-stranded DNA. *J. Virol.* **72**, 9247-9256.

Qin, Y. & Petty, I. T. (2001). Genetic analysis of bipartite geminivirus tissue tropism. *Virology* **291**, 311-323.

Rasheed, M. S., Selth, L. A., Koltunow, A. M., Randles, J. W. & Rezaian, M. A. (2006). Single-stranded DNA of Tomato leaf curl virus accumulates in the cytoplasm of phloem cells. *Virology* **348**, 120-132.

Ratcliff, F., Harrison, B. D. & Baulcombe, D. C. (1997). A similarity between viral defence and gene silencing in plants. *Science* **276**, 1558-1566.

Reichel, C., Mas, P. & Beachy, R. N. (1999). The role of the ER and cytoskeleton in plant viral trafficking. *Trends Plant Sci.* **4**, 458-462.

Rezaian, M. A., Krake, L. R., Cunying, Q. & Hazzalin, C. A. (1991). Detection of virus-associated dsRNA from leafroll infected grapevines. *J. Virol. Methods* **31**, 325-334.

Rigden, J. E., Dry, I. B., Krake, L. R. & Rezaian, M. A. (1996). Plant virus DNA replication processes in Agrobacterium: insight into the origins of geminiviruses? *Proc. Natl. Acad. Sci. USA* **93**, 10280-10284.

Rigden, J. E., Dry, I. B., Mullineaux, P. M. & Rezaian, M. (1993). Mutagenesis of the virion-sense open reading frames of tomato leaf curl geminivirus. *Virology* **193**, 1001-1005.

Rigden, J. E., Krake, L. R., Rezaian, M. A. & Dry, I. B. (1994). ORF C4 of tomato leaf curl geminivirus is a determinant of symptom severity. *Virology* **204**, 847-850.

Ritzenthaler, C., Nebenfuhr, A., Movafeghi, A., Stussi-Garaud, C., Behnia, L., Pimpl, P., Staehelin, L. A. & Robinson, D. G. (2002). Reevaluation of the effects of brefeldin A on plant cells using tobacco Bright Yellow 2 cells expressing Golgi-targeted green fluorescent protein and COPI antisera. *Plant Cell* **14**, 237-261.

Robards, A. W. & Lucas, W. J. (1990). Plasmodesmata. *Annu. Rev. Plant Physiol.* **41**, 369-419.

Roberts, A. G., Cruz, S. S., Roberts, I. M., Prior, D., Turgeon, R. & Oparka, K. J. (1997). Phloem Unloading in Sink Leaves of Nicotiana benthamiana: Comparison of a Fluorescent Solute with a Fluorescent Virus. *Plant Cell* **9**, 1381-1396.

Roberts, I. M., Boevink, P., Roberts, A. G., Sauer, N., Reichel, C. & Oparka, K. J. (2001). Dynamic changes in the frequency and architecture of plasmodesmata during the sink-source transition in tobacco leaves. *Protoplasma* **218**, 31-44.

Rodman, M. K., Yadav, N. S. & Artus, N. N. (2002). Progression of geminivirus-induced transgene silencing is associated with transgene methylation. *New Phytol.* **155**, 461-486.

Rojas, M. R., Hagen, C., Lucas, W. J. & Gilbertson, R. L. (2005). Exploiting Chinks in the Plant's Armor: Evolution and Emergence of Geminiviruses. *Annu. Rev. Phytopathol.* **43**, 361-394.

Rojas, M. R., Jiang, H., Salati, R., Xoconostle-Cazares, B., Sudarshana, M. R., Lucas, W. J. & Gilbertson, R. L. (2001). Functional analysis of proteins involved in movement of the monopartite begomovirus, Tomato yellow leaf curl virus. *Virology* **291**, 110-25.

Rojas, M. R., Noueiry, A. O., Lucas, W. J. & Gilbertson, R. L. (1998). Bean dwarf mosaic geminivirus movement proteins recognize DNA in a form- and size-specific manner. *Cell* **95**, 105-113.

Rushing, A. E., Sunter, G., Gardiner, W. E., Dute, R. R. & Bisaro, D. M. (1987). Ultrastructural aspects of tomato golden mosaic virus infection in tobacco. *Phytopathology* **77**, 1231-1236.

Ryabov, E. V., Oparka, K. J., Santa Cruz, S., Robinson, D. J. & Taliansky, M. E. (1998). Intracellular location of two groundnut rosette umbravirus proteins delivered by PVX and TMV vectors. *Virology* **242**, 303-313.

Saeed, M., Behjatnia, S. A., Mansoor, S., Zafar, Y., Hasnain, S. & Rezaian, M. A. (2005). A single complementary-sense transcript of a geminiviral DNA beta satellite is determinant of pathogenicity. *Mol. Plant-Microbe Interact.* **18**, 7-14.

Sagi, G., Katz, A., Guenoune-Gelbart, D. & Epel, B. L. (2005). Class 1 reversibly glycosylated polypeptides are plasmodesmal-associated proteins delivered to plasmodesmata via the golgi apparatus. *Plant Cell* **17**, 1788-1800.

Saikia, A. K. & Muniyappa, V. (1989). Epidemiology and control of tomato leaf curl virus in southern India. *Trop. Agric.* **66**, 350-354.

Sambrook, J. & Russell, D. W. (2001). Molecular Cloning: A Laboratory Manual, 3rd edn. New York: Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

Sanderfoot, A. A., Ingham, D. J. & Lazarowitz, S. G. (1996). A viral movement protein as a nuclear shuttle. The geminivirus BR1 movement protein contains domains essential for interaction with BL1 and nuclear localization. *Plant Physiol.* **110**, 23-33.

Sanderfoot, A. A. & Lazarowitz, S. G. (1995). Cooperation in viral movement: the geminivirus BL1 movement protein interacts with BR1 and redirects it from the nucleus to the cell periphery. *Plant Cell* **7**, 1185-1194.

Sanderfoot, A. A. & Lazarowitz, S. G. (1996). Getting it together in plant virus movement: Cooperative interactions between bipartite geminivirus movement proteins. *Trends Cell Biol.* **6**, 353-385.

Saunders, K., Bedford, I. D., Briddon, R. W., Markham, P. G., Wong, S. M. & Stanley, J. (2000). A unique virus complex causes Ageratum yellow vein disease. *Proc. Natl. Acad. Sci. USA* **97**, 6890-6895.

Saunders, K., Bedford, I. D. & Stanley, J. (2001a). Pathogenicity of a natural recombinant associated with ageratum yellow vein disease: implications for geminivirus evolution and disease aetiology. *Virology* **282**, 38-47.

Saunders, K., Bedford, I. D., Yahara, T. & Stanley, J. (2003). The earliest recorded plant virus disease. *Nature* **422**, 831.

Saunders, K., Lucy, A. & Stanley, J. (1991). DNA forms of the geminivirus African cassava mosaic virus consistent with a rolling circle mechanism of replication. *Nucleic Acids Res.* **19**, 2325-2330.

Saunders, K., Norman, A., Gucciardo, S. & Stanley, J. (2004). The DNA beta satellite component associated with ageratum yellow vein disease encodes an essential pathogenicity protein (betaC1). *Virology* **324**, 37-47.

Saunders, K., Salim, N., Mali, V. R., Malathi, V. G., Briddon, R., Markham, P. G. & Stanley, J. (2002). Characterisation of Sri Lankan cassava mosaic virus and Indian cassava mosaic virus: evidence for acquisition of a DNA B component by a monopartite begomovirus. *Virology* **293**, 63-74.

Saunders, K. & Stanley, J. (1999). A nanovirus-like DNA component associated with yellow vein disease of Ageratum conyzoides: evidence for interfamilial recombination between plant DNA viruses. *Virology* **264**, 142-152.

Saunders, K., Wege, C., Veluthambi, K., Jeske, H. & Stanley, J. (2001b). The distinct disease phenotypes of the common and yellow vein strains of Tomato golden mosaic virus are determined by nucleotide differences in the 3'-terminal region of the gene encoding the movement protein. *J. Gen. Virol.* **82**, 45-51.

Schauer, S. E., Jacobsen, S. E., Meinke, D. W. & Ray, A. (2002). DICER-LIKE1: blind men and elephants in Arabidopsis development. *Trends Plant Sci.* **7**, 487-491.

Schenborn, E. T. & Mierendorf, R. C., Jr. (1985). A novel transcription property of SP6 and T7 RNA polymerases: dependence on template structure. *Nucleic Acids Res.* **13**, 6223-6236.

Schneider, I. R. & Worley, J. F. (1959). Rapid entry of infectious particles of southern bean mosaic virus into living cells following transport of the particles in the water stream. *Virology* **8**, 243-249.

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

Selth, L. A., Dogra, S. C., Rasheed, M. S., Healy, H., Randles, J. W. & Rezaian, M. A. (2005). A NAC Domain Protein Interacts with Tomato leaf curl virus Replication Accessory Protein and Enhances Viral Replication. *Plant Cell* **17**, 311-325.

Selth, L. A., Dogra, S. C., Rasheed, M. S., Randles, J. W. & Rezaian, M. A. (2006). Identification and Characterization of a Host Reversibly Glycosylated Peptide that Interacts with the Tomato leaf curl virus V1 Protein. *Plant Mol. Biol.* **61**, 297-310.

Selth, L. A., Randles, J. W. & Rezaian, M. A. (2002). Agrobacterium tumefaciens supports DNA replication of diverse geminivirus types. *FEBS Lett.* **516**, 179-182.

Selth, L. A., Randles, J. W. & Rezaian, M. A. (2004). Host responses to transient expression of individual genes encoded by Tomato leaf curl virus. *Mol. Plant-Microbe Interact.* **17**, 27-33.

Settlage, S. B., Miller, A. B., Gruissem, W. & Hanley-Bowdoin, L. (2001). Dual interaction of a geminivirus replication accessory factor with a viral replication protein and a plant cell cycle regulator. *Virology* **279**, 570-576.

Sijen, T., Vijn, I., Rebocho, A., van Blokland, R., Roelofs, D., Mol, J. N. & Kooter, J. M. (2001). Transcriptional and posttranscriptional gene silencing are mechanistically related. *Curr. Biol.* **11**, 436-440.

Smith, N. A., Singh, S. P., Wang, M. B., Stoutjesdijk, P. A., Green, A. G. & Waterhouse, P. M. (2000). Total silencing by intron-spliced hairpin RNAs. *Nature* **407**, 319-320.

Smith, S. S., Kaplan, B. E., Sowers, L. C. & Newman, E. M. (1992). Mechanism of human methyl-directed DNA methyltransferase and the fidelity of cytosine methylation. *Proc. Natl. Acad. Sci. USA* **89**, 4744-4748.

Stanley, J. & Latham, J. R. (1992). A symptom variant of beet curly top geminivirus produced by mutation of open reading frame C4. *Virology* **190**, 506-509.

Stanley, J. & Townsend, R. (1985). Characterisation of DNA forms associated with cassava latent virus infection. *Nucleic Acids Res.* **13**, 2189-2206.

Stenger, D. C. (1994). Strain-specific mobilization and amplification of a transgenic defective-interfering DNA of the geminivirus beet curly top virus. *Virology* **203**, 397-402.

Stenger, D. C., Stevenson, M. C., Hormuzdi, S. G. & Bisaro, D. M. (1992). A number of subgenomic DNAs are produced following agroinoculation of plants with beet curly top virus. *J. Gen. Virol.* **73**, 237-242.

Stone, B. A. & Clarke, A. E. (1992). Chemistry and biology of (1-3)-ß- glucans. Victoria, Australia: La Trobe University Press.

Stonor, J., Hart, P., Gunther, M., DeBarro, P. & Rezaian, M. A. (2003). Tomato leaf curl geminivirus in Australia: occurrence, detection, sequence diversity and host range. *Plant Pathol.* **52**, 379-388.

Sunter, G. & Bisaro, D. M. (1991). Transactivation in a geminivirus; AL2 gene product is needed for coat protein expression. *Virology* **180**, 416-419.

Sunter, G., Hartitz, M. D., Hormuzdi, S. G., Brough, C. L. & Bisaro, D. M. (1990). Genetic analysis of tomato golden mosaic virus: ORF AL2 is required for coat protein accumulation while ORF AL3 is necessary for efficient DNA replication. *Virology* **179**, 69-77.

Sunter, G., Sunter, J. L. & Bisaro, D. M. (2001). Plants expressing tomato golden mosaic virus AL2 or beet curly top virus L2 transgenes show enhanced susceptibility to infection by DNA and RNA viruses. *Virology* **285**, 59-70.

Taliansky, M. & Barker, H. (1999). Movement of luteoviruses in infected plants. In *The Luteoviridae*, pp. 69-81. Edited by H. G. Smith & H. Barker. Wallingford, UK: CAB International.

Tang, W. & Leisner, S. (1998). Methylation of nonintegrated multiple copy DNA in plants. *Biochem. Biophys. Res. Commun.* **245**, 403-406.

Tao, X. & Zhou, X. (2004). A modified viral satellite DNA that suppresses gene expression in plants. *Plant J.* **38**, 850-860.

Tao, X. R., Qing, L. & Zhou, X. P. (2004). Function of A-rich region in DNA ßassociated with Tomato leaf curl China virus. *Chin. Sci. Bull.* **49**, 1490-1493.

Thommes, P., Osman, T. A. M., Hayes, R. J. & Buck, K. W. (1993). TGMV replication protein AL1 preferentially binds to single-stranded DNA from the common region. *FEBS Lett.* **319**, 95-99.

Townsend, R., Stanley, J., Curson, S. J. & Short, M. N. (1985). Major polyadenylated transcripts of cassava latent virus and location of the gene encoding coat protein. *EMBO J.* **4**, 33-37.

Turner, A., Wells, B. & Roberts, K. (1994). Plasmodesmata of maize root tips: structure and composition. *J. Cell Sci.* **107**, 3351-3361.

Unseld, S., Frischmuth, T. & Jeske, H. (2004). Short deletions in nuclear targeting sequences of African cassava mosaic virus coat protein prevent geminivirus twinned particle formation. *Virology* **318**, 90-101.

van der Krol, A. R., Mur, L. A., Beld, M., Mol, J. N. & Stuitje, A. R. (1990). Flavonoid genes in *Petunia*: addition of a limited number of gene copies may lead to a suppression of gene expression. *Plant Cell* **2**, 291-299.

Van Houdt, H., Bleys, A. & Depicker, A. (2003). RNA target sequences promote spreading of RNA silencing. *Plant Physiol.* **131**, 245-253.

van, W. R., Dong, X., Liu, H., Tien, P., Stanley, J. & Hong, Y. (2002). Mutation of three cysteine residues in Tomato yellow leaf curl virus-China C2 protein causes dysfunction in pathogenesis and posttranscriptional gene-silencing suppression.PG - 203-8. *Mol. Plant-Microbe Interact.* **15**, 203-208.

Vanitharani, R., Chellappan, P., Pita, J. S. & Fauquet, C. M. (2004). Differential roles of AC2 and AC4 of cassava geminiviruses in mediating synergism and suppression of posttranscriptional gene silencing. *J. Virol.* **78**, 9487-9498.

Varma, A. & Malathi, V. G. (2003). Emerging geminivirus problems: A serious threat to crop production. *Ann. Appl. Biol.* **142**, 145-164.

Verchot, J., Driskel, B. A., Zhu, Y., Hunger, R. M. & Littlefield, L. J. (2001). Evidence that soilborne wheat mosaic virus moves long distance through the xylem in wheat. *Protoplasma* **218**, 57-66.

Voinnet, O. (2001). RNA silencing as a plant immune system against viruses. *Trends Genet* **17**, 449-459.

Voinnet, O., Pinto, Y. M. & Baulcombe, D. C. (1999). Suppression of gene silencing: a general strategy used by diverse DNA and RNA viruses of plants. *Proc. Natl. Acad. Sci. USA* **96**, 14147-14152.

von Arnim, A. & Stanley, J. (1992). Determinants of tomato golden mosaic virus symptom development located on DNA B. *Virology* **186**, 286-293.

Wang, H., Hao, L. H., Shung, C. Y., Sunter, G. & Bisaro, D. M. (2003). Adenosine kinase is inactivated by geminivirus AL2 and L2 proteins. *Plant Cell* **15**, 3020-3032.

Wang, H. L., Gilbertson, R. L. & Lucas, W. J. (1996). Spatial and temporal distribution of bean dwarf mosaic geminivirus in Phaseolus vulgaris and Nicotiana benthamiana. *Phytopathology* **86**, 1204-1214.

Wang, M. B., Wesley, S. V., Finnegan, E. J., Smith, N. A. & Waterhouse, P. M. (2001). Replicating satellite RNA induces sequence-specific DNA methylation and truncated transcripts in plants. *RNA* **7**, 16-28.

Ward, B. M. & Lazarowitz, S. G. (1999). Nuclear export in plants: use of geminivirus movement proteins for a cell-based export assay. *Plant Cell* **11**, 1267-1276.

Ward, B. M., Medville, R., Lazarowitz, S. G. & Turgeon, R. (1997). The geminivirus BL1 movement protein is associated with endoplasmic reticulum-derived tubules in developing phloem cells. *J. Virol.* **71**, 3726-3733.

Wartig, L., Kheyr-Pour, A., Noris, E., Kouchkovsky, F. d., Jouanneau, F., Gronenborn, B., Jupin, I. & De, K. o. (1997). Genetic analysis of the monopartite tomato yellow leaf curl geminivirus: roles of V1, V2, and C2 ORFs in viral pathogenesis. *Virology* **228**, 132-140.

Waterhouse, P. M., Smith, N. A. & Wang, M. B. (1999). Virus resistance and gene silencing: killing the messenger. *Trends Plant Sci.* **4**, 452-457.

Waterhouse, P. M., Wang, M. B. & Lough, T. (2001). Gene silencing as an adaptive defence against viruses. *Nature* **411**, 834-842.

Wege, C. & Jeske, H. (1998). Abutilon mosaic geminivirus proteins expressed and phosphorylated in Escherichia coli. *J. Phytopathol.* **146**, 613-621.

Wege, C., Saunders, K., Stanley, J. & Jeske, H. (2001). Comparative analysis of tissue tropism of bipartite geminiviruses. *J. Phytopathol.* **149**, 359-368.

Wilkinson, D. G. (1992). *In situ* hybridization: A practical approach. Edited by D. G. Wilkinson. Oxford: IRL Press.

Wilkinson, D. G. & Green, J. (1990). *In situ* hybridization to cellular RNA and the three-dimensional reconstruction of serial sections. In *Post-implantation Mammalian Development*, pp. 155-171. Edited by A. Copp & D. Cockcroft. Oxford: IRL Press.

Yang, Y., Sherwood, T. A., Patte, C. P., Hiebert, E. & Polston, J. E. (2004). Use of Tomato yellow leaf curl virus (TYLCV) rep gene sequences to engineer TYLCV resistance in tomato. *Phytopathol.* **94**, 490-496.

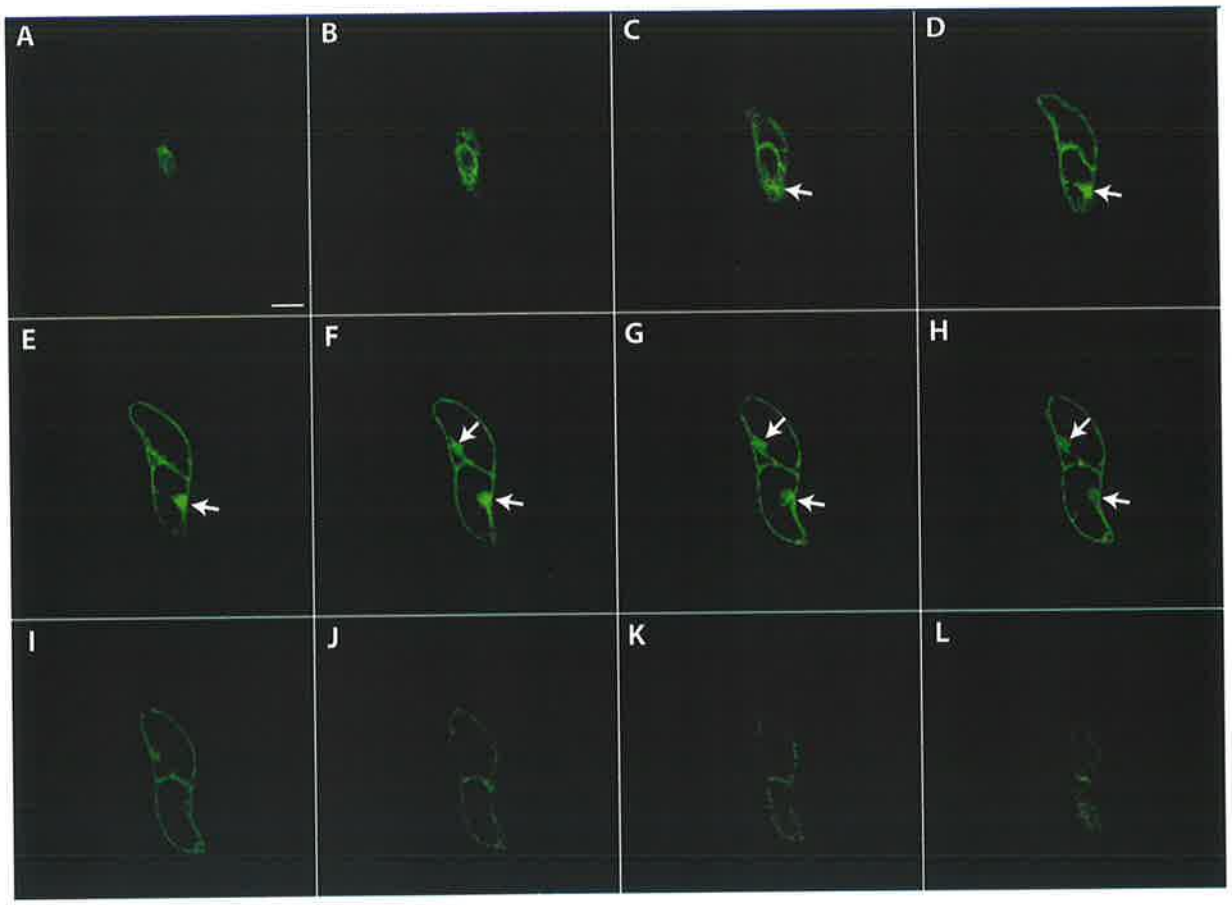
Zambryski, P. & Crawford, K. (2000). Plasmodesmata: gatekeepers for cell-to-cell transport of developmental signals in plants. *Annu. Rev. Cell Dev. Biol.* **16**, 393-421.

Zamore, P. D., Tuschl, T., Sharp, P. A. & Bartel, D. P. (2000). RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. *Cell* **101**, 25-33.

Zhang, S. C., Wege, C. & Jeske, H. (2001). Movement proteins (BC1 and BV1) of Abutilon mosaic geminivirus are cotransported in and between cells of sink but not of source leaves as detected by green fluorescent protein tagging. *Virology* **290**, 249-260.

Appendix 1 – Nuclear localization of V1:GFP

Serial optical layers of image shown in Fig. 5.4A:



Appendix-1 Serial optical sections of image showing in Fig. 5.4A show nuclear localization of V1:GFP indicated by white arrows **(C-H).** Bar = 25 µm.

Appendix 2 – Cover illustrations

Images from work in this thesis have been used for cover illustrations of two journals:

1) Virology : Volume 348, Issue 1, April 2006
2) MPMI: Volume 19, Issue 6, June 2006

Cover legend:

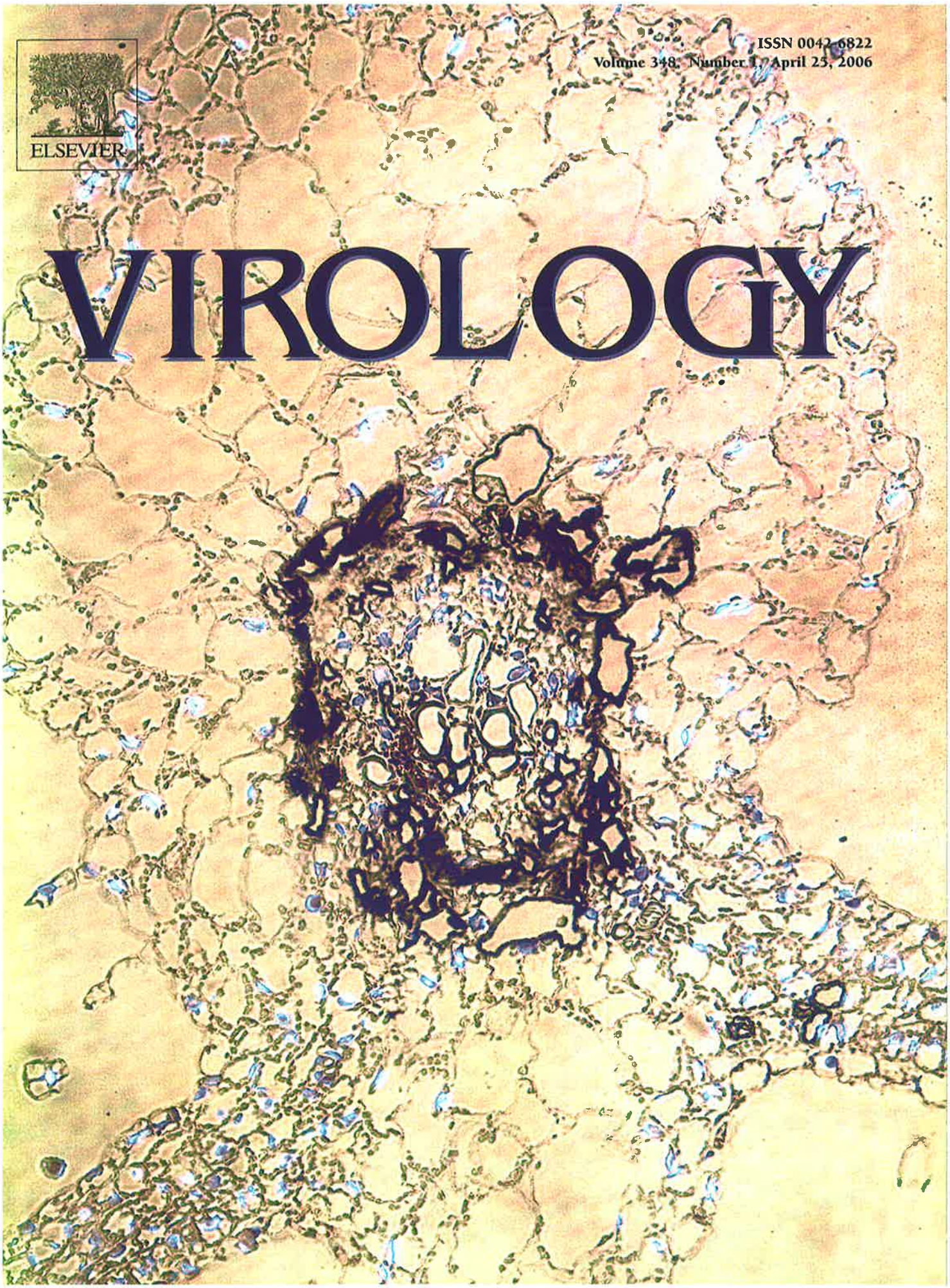
Geminiviruses contain a ssDNA genome and replicate in the nuclei of plant cells via replicative dsDNA. Tissue tropism and intracellular localization of different forms of *Tomato leaf curl virus* in a section of leaf vein are shown. Contrary to the current understanding of geminiviral localization, the viral single-stranded DNA accumulated in the cytoplasm of vascular cells as shown by dark blue signals. Nuclei of both vascular and non-vascular cells are indicated by light blue color. See the article by Rasheed et al. in this issue.

(Virology : Volume 348, Issue 1, April 2006)

ISSN 0042-6822
Volume 348, Number 1, April 25, 2006

ELSEVIER

VIROLOGY



Cover legend:

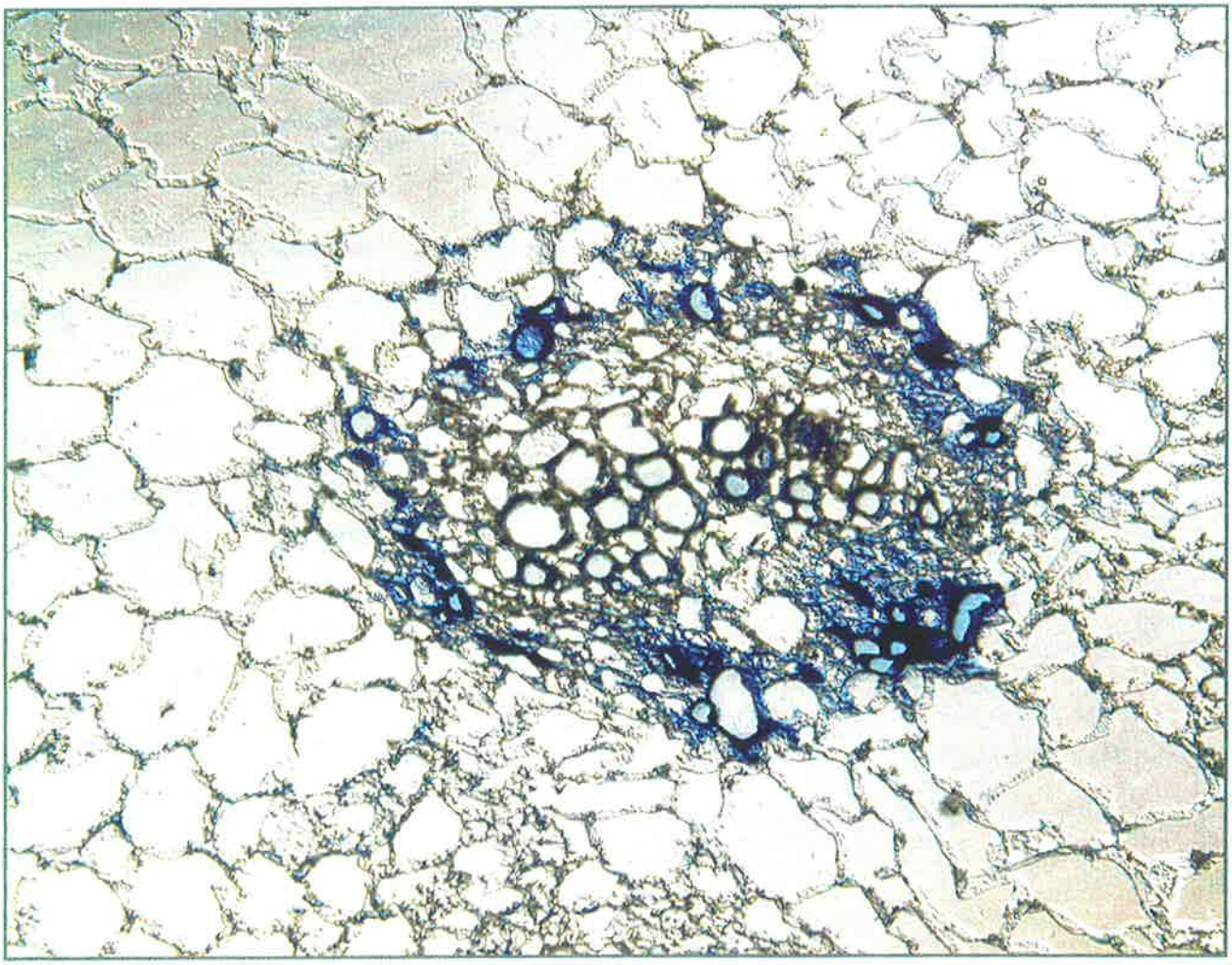
Tomato leaf vurl virus (TLCV) is a member of the *Geminiviridae* and contains a ssDNA genome. A section of tobacco leaf tissue carrying the TLCV *C4* transgene is shown. Upon infection, the transgene expression disappears from the nonvascular cells while the virus escapes silencing, as seen by its accumulation in the vascular tissues (blue signals). For the article by Bian et al., see page 614 of this issue.

(MPMI: Volume 19, Issue 6, June 2006)

MPMI

Molecular Plant-Microbe Interactions®

VOLUME 19, NUMBER 6 ■ June 2006 ■ ISSN 0894-0282



Appendix 3 - Publications arising from this work

Sections of this thesis have been published in the following papers:

Selth, L. A., Dogra, S. C., Rasheed, M. S., Healy, H., Randles, J. W., & Rezaian, M. A. (2005). A NAC Domain Protein Interacts with Tomato leaf curl virus Replication Accessory Protein and Enhances Viral Replication. *The Plant Cell*, *17*(1), 311-325.

Available online at www.sciencedirect.com
SCIENCE DIRECT
ELSEVIER
Virology 348 (2006) 120–132
VIROLOGY
www.elsevier.com/locate/yviro

Single-stranded DNA of *Tomato leaf curl virus* accumulates in the cytoplasm of phloem cells

M. Saif Rasheed [a,b,c], Luke A. Selth [a,b], Anna M.G. Koltunow [a], John W. Randles [b], M. Ali Rezaian [a,*]

[a] *CSIRO Plant Industry, P.O. Box 350, Glen Osmond, SA 5064, Australia*
[b] *School of Agriculture and Wine, The University of Adelaide, Adelaide, SA 5005, Australia*
[c] *National Institute for Biotechnology and Genetic Engineering (NIBGE), P.O. Box 577, Jhang Road, Faisalabad, Pakistan*

Received 8 September 2005; returned to author for revision 9 November 2005; accepted 11 November 2005
Available online 2 February 2006

Abstract

Geminiviruses have been reported to replicate in, and localize to, the nuclei of host plant cells. We have investigated the tissue and intracellular distribution of the monopartite *Tomato leaf curl virus* (TLCV) by in situ hybridization. Contrary to the current understanding of geminiviral localization, single-stranded (ss) DNA of TLCV accumulated in the cytoplasm. TLCV ssDNA was also found in the nucleus, as was lower levels of replicative form double-stranded (ds) DNA. Under the same conditions, *Tomato golden mosaic virus* (TGMV) ssDNA and dsDNA were found in nuclei. ssDNA of TLCV, TGMV, and *Tomato yellow leaf curl Sardinia virus* (TYLCSV) was detected in some xylem vessels under specific hybridization conditions. Tissue specificity of TLCV was partially released by co-infection with TGMV. Our observations suggest that the mechanism of TLCV movement may differ from that of bipartite begomoviruses.

Keywords: Geminivirus; Begomovirus; ssDNA; Cytoplasm; Phloem-limited virus; Dual color in situ hybridization; Xylem; TLCV

Introduction

Geminiviruses (family: Geminiviridae) possess either one or two circular, single-stranded (ss) DNA genomes of 2.5–3 kb. They replicate in plant cell nuclei via double-stranded (ds) DNA intermediates (Gutierrez, 2000; Hanley-Bowdoin et al., 1999). Geminiviruses have been classified into four genera, *Begomovirus*, *Curtovirus*, *Mastrevirus*, and *Topocuvirus*, based on their genome organization, host range, and insect vector (Stanley et al., 2005). Begomoviruses are whitefly transmitted and contain either monopartite or bipartite DNA genomes.

All previous studies of geminiviral DNA localization have indicated viral nucleic acid exclusively in the nucleus of infected cells. For example, *Tomato golden mosaic virus* (TGMV) infection led to the accumulation of virions, often as paracrystalline arrays, in nuclei of both vascular and non-vascular cells of *Nicotiana benthamiana* (Rushing et al., 1987). Bass et al. (2000) observed altered nuclear architecture and plant chromatin condensation upon TGMV infection, indicating that viral DNA was present in the nucleus. Moreover, studies of the tissue specificity of both monopartite (Morilla et al., 2004; Rojas et al., 2001) and bipartite (Morra and Petty, 2000; Qin and Petty, 2001; Wege et al., 2001) geminiviruses by in situ hybridization showed that viral DNA accumulated in the nuclei of infected cells.

To date, the understanding of monopartite geminivirus movement is mostly based on studies of their bipartite counterparts. Monopartite begomoviruses possess six open reading frames (ORFs), two on the virion-sense strand and four on the complementary-sense strand (Hanley-Bowdoin et al., 1999). In contrast to the bipartite begomoviruses, the coat protein (CP) of monopartite begomoviruses is essential for systemic infection (Noris et al., 1998; Rigden et al., 1993) and appears to act analogously to the nuclear shuttle protein (NSP) of bipartite begomoviruses (Rojas et al., 2001). Two other proteins from monopartite begomoviruses, V1 and C4, have been implicated in cell-to-cell movement and may have a functional similarity to the movement protein (MP) of bipartite geminiviruses (Rigden et al., 1993; Rojas et al., 2001).

* Corresponding author. Fax: +61 8 8303 8601.
E-mail address: ali.rezaian@csiro.au (M.A. Rezaian).

0042-6822/$ - see front matter.
doi:10.1016/j.virol.2005.11.054

The reasons why some viruses are restricted to vasculature while others are capable of infecting the mesophyll remain unclear. It has been suggested that vascular-restricted infection might occur because viral MPs are unable to function in epidermal and mesophyll cells (Taliansky and Barker, 1999). Another hypothesis is that some viruses cannot suppress host defence responses in non-vascular cells (Voinnet et al., 1999; Waterhouse et al., 1999). The observation that vascular limitation of phloem-restricted viruses can be overcome by co-infection with mesophyll-invasive viruses supports this latter idea (Morra and Petty, 2000; Wege et al., 2001). Tissue specificity of a virus can also be affected by environmental factors (Ding et al., 1999) and the developmental stage of the plant (Wang et al., 1996).

It has been suggested that geminiviruses move from cell-to-cell and systemically either in the form of virions or in nucleoprotein complexes (Gafni and Bernard, 2002). However, the form of viral DNA involved in viral trafficking is unknown. The NSP and MP of *Bean dwarf mosaic virus* (BDMV), a mesophyll-invasive bipartite begomovirus, bind ss- and dsDNA in a form- and size-selective manner (Rojas et al., 1998), and dsDNA is the predominant form of viral cargo (Noueiry et al., 1994; Rojas et al., 1998). In contrast, the NSP from *Squash leaf curl virus* (SqLCV), a phloem-limited bipartite begomovirus, binds strongly to ssDNA but weakly to dsDNA, while the MP associates weakly with ssDNA and does not appear to bind dsDNA at all (Pascal et al., 1994). A phage protein (M13; g5p) which binds viral ssDNA inhibited the movement of *Tomato leaf curl New Delhi virus* (ToLCNDV), suggesting that ssDNA moves from cell-to-cell in ToLCNDV infection (Padidam et al., 1999). It has been proposed that different tissue tropisms exhibited by bipartite geminiviruses might be the result of different movement mechanisms (Morra and Petty, 2000).

We describe here the intracellular localization of ssDNA and dsDNA of the monopartite begomovirus Australian *Tomato leaf curl virus* (TLCV) in comparison with that of the bipartite begomovirus, TGMV, in both single and mixed infections. Our in situ hybridization studies indicate that ssDNA could play a major role in TLCV trafficking through the cytoplasm.

Results

Specificity of riboprobes to detect targeted viral nucleic acid forms

To identify the different viral DNA species at the subcellular level using in situ hybridization, we used a series of digoxigenin (DIG)- and fluorescein-labeled probes corresponding to different regions of TLCV and TGMV DNA (Fig. 1A and Table 1). The ability of probes to differentiate single-stranded and double-stranded replicative form DNA was tested by DNA gel blot hybridization in a preliminary experiment. As shown in Fig. 1B, the probes hybridized with dsDNA replicative forms and ssDNA in a strand-specific manner. A larger amount of ssDNA was detected compared to other forms of viral DNA using complementary-sense probes specific for coat protein, replication associated protein and intergenic region (Fig. 1B, Table 1). It is worth noting that a small amount of DNA corresponding to complementary-sense (cs) ssDNA was also visible in lanes 1 and 5 using virion-sense probes (Fig. 1B). A relatively low level of cs ssDNA has also been reported in *African cassava mosaic virus* infection (Saunders et al., 1991). Furthermore, subgenomic DNAs were found in *N. benthamiana* plants agroinoculated with TLCV but not in tomato. Moreover, subgenomic DNAs were not found in *N. benthamiana* plants agroinoculated with TGMV (Fig. 1B).

The specificity of the probes was tested by hybridization to tissue sections from a mock-inoculated *N. benthamiana* plant. No signals were observed in the mock-inoculated sections (Fig. 1C1), indicating that non-specific binding to the sections was not occurring. The TGMV- or TLCV-specific probes designed to target viral DNA and RNA transcripts (CS-cp; Table 1) produced strong chromogenic signals in leaf sections infected with the respective viruses (Figs. 1C4 and C5). Since diseased plants may have a different physiology from healthy plants, we tested whether signals were due to background reaction with stress factors by hybridizing sections from diseased plants with probes designed to target the heterologous virus. No signals were observed in sections of a TLCV-infected plant probed with TGMV-specific CS-cp RNA (Fig. 1C2) or in sections taken from a TGMV-infected plant hybridized with TLCV-specific CS-cp probe (Fig. 1C3). These results confirmed that the in situ hybridization procedure was specific for detecting viral nucleic acids.

Using in situ hybridization, TLCV was found only in the vascular tissue of systemically infected *N. benthamiana* leaves (Figs. 1C4 and 2A–I). Infected cells were more abundant in young symptomatic leaves than in developing and mature leaves (data not shown). In the transverse section of a *N. benthamiana* leaf vein (Figs. 1C4 and 2A), TLCV was associated with phloem parenchyma, sieve elements, companion cells, vascular cambium, and extended to the bundle sheath cells but was not found in other cell types. Phloem, xylem, and sieve elements are shown in a longitudinal section taken from leaf vein of TLCV-infected *N. benthamiana* (Fig. 2D). The identity of these cell types was determined by staining the sections with 0.1% toluidine blue (data not shown) (Mauseth, 1988). A similar tissue tropism was found in lateral root (Fig. 2G), stem (Fig. 2H), and leaf (Fig. 2I) tissue. In the stem section (Fig. 2H), the virus was associated with both inner and outer vascular cells and some adjacent cells in the cortex and pith. Vascular specificity of TLCV was also observed in *Solanum lycopersicum* and *N. tabacum*, although chromogenic signals were not as strong as in *N. benthamiana* (Figs. 2E and F). Unlike TLCV, TGMV was detectable in both vascular and non-vascular cells (Figs. 1C5 and 2J–L), as reported earlier (Morra and Petty, 2000; Nagar et al., 1995; Wege et al., 2001).

TLCV nucleic acid accumulates in the cytoplasm

The majority of phloem cells produced strong hybridization signals. These signals indicated the presence of TLCV nucleic acid in both cytoplasm and nucleus (Figs. 1C4 and 2A). Identity of the cytoplasm in fixed cells was confirmed by probing the

healthy sections with a ubiquitin probe to detect ubiquitin transcripts (Fig. 3C). Nuclei were visualized by DAPI (4′,6-diamidino-2-phenylindole dihydrochloride) treatment and were detected in the majority of cells (Fig. 2B). Absence of signals from some cells was presumably because nuclei occupy a small portion of the cell volume and therefore may have not been

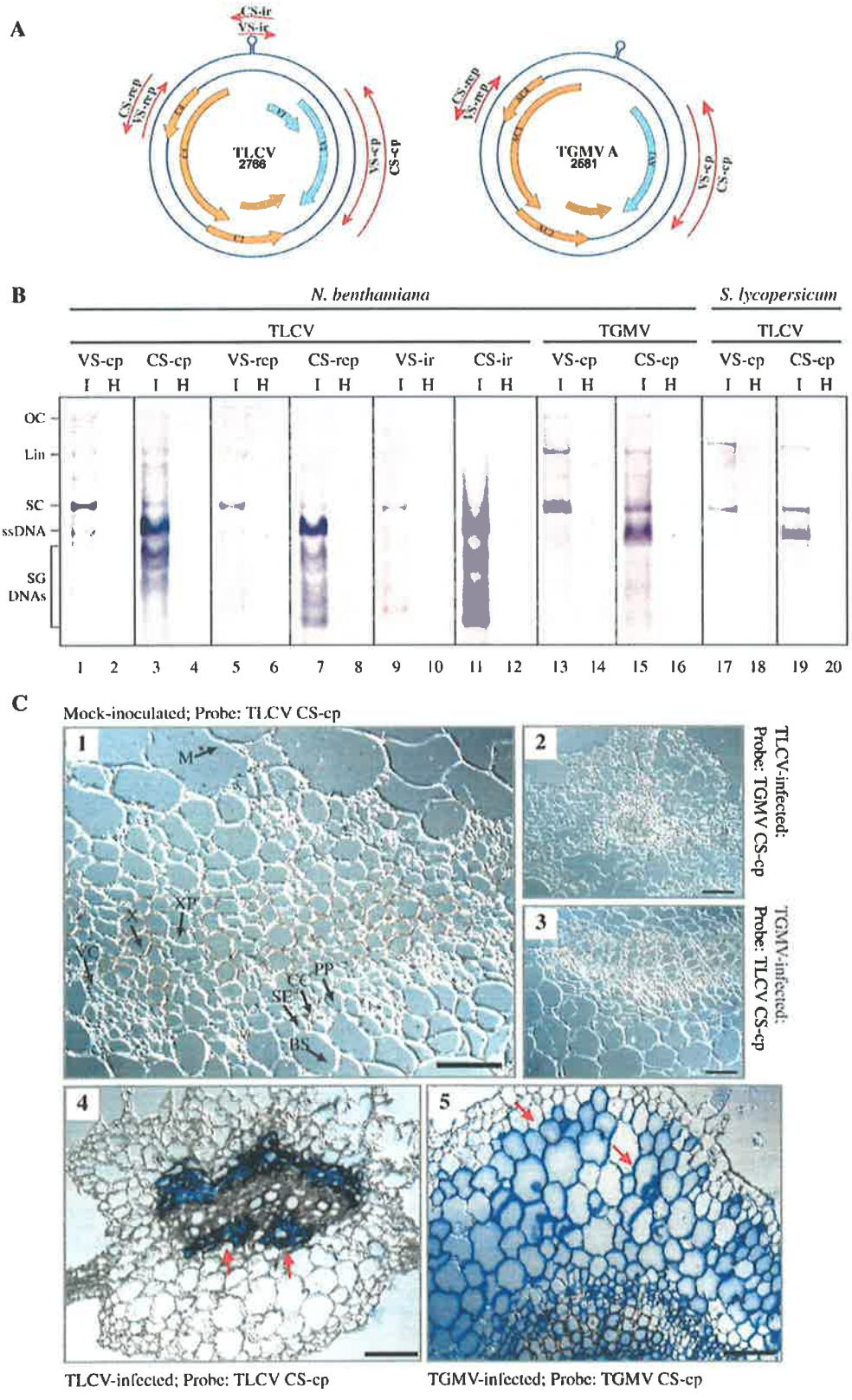


Table 1
TLCV, TGMV, and TYLCSV specific ribonucleotide probes

Probes[a]	Source	Nucleotide position[b]	Size (nt)	Potential targets
VS-cp	TLCV	308–1078	771	dsDNA
CS-cp	TLCV	1078–308	771	ss and dsDNA, CP transcript
VS-rep	TLCV	2156–2464	309	dsDNA and Rep transcript
CS-rep	TLCV	2464–2156	309	ss and dsDNA
VS-ir	TLCV	2656–127	238	dsDNA
CS-ir	TLCV	127–2656	238	ss and dsDNA
VS-cp	TGMV	327–1070	744	dsDNA
CS-cp	TGMV	1070–327	744	ss and dsDNA, CP transcript
VS-rep	TGMV	1962–2210	249	dsDNA and Rep transcript
CS-rep	TGMV	2210–1962	249	ss and dsDNA
VS-rep	TYLCSV	2170–2466	309	dsDNA and Rep transcript
CS-rep	TYLCSV	2466–2170	309	ss and dsDNA
CS-ubi	Ubiquitin	–	–	Ubiquitin transcript

[a] VS, virion sense; CS, complementary sense; cp, coat protein; rep, replication protein; ir, intergenic region; ubi, ubiquitin.
[b] Nucleotide position for TLCV as in Dry et al. (1993), for TGMV as in von Arnim and Stanley (1992) and for TYLCSV as in Kheyr-Pour et al. (1991).

represented in the respective section. DAPI staining was masked by the strong chromogenic signals produced by the DIG-labeled probe (Fig. 2C). Merging of images obtained with the TLCV-specific probe and DAPI staining confirmed that viral nucleic acid was not confined to the nucleus only but was also present in the cytoplasm (Fig. 2C).

Strong chromogenic signals in the cytoplasm of TLCV infected cells were obtained with the CS-cp probe (Figs. 1C4 and 2A–I). This probe could detect both viral DNA and virion-sense transcripts (Table 1). To distinguish viral DNA from transcripts, we removed RNA enzymatically. Thus two serial sections from TLCV-infected tissue were prepared, and one was treated with DNase-free RNase A in a NaCl-deficient buffer to digest both ss- and dsRNA (Maththews, 1993; Rezaian et al., 1991). These sections were hybridized with the homologous TLCV-specific CS-cp probe. RNase treated, TLCV-infected sections still exhibited chromogenicity in the cytoplasm (Figs. 3A and D). Ubiquitin mRNA in healthy sections was used as a control to verify the effectiveness of RNase treatment and to ensure that residual RNase was not interfering with hybridization of the riboprobes. The ubiquitin signal detected in healthy sections (Fig. 3C) was lost after RNase treatment (Fig. 3F). Similarly, signals were retained in the nuclei of cells in sections derived from TGMV-infected plants after RNase treatment (Fig. 3E). These results are consistent with previous findings (Morra and Petty, 2000; Qin and Petty, 2001; Wege et al., 2001) that TGMV viral DNA is localized in nuclei (Fig. 3E), while the transcripts are present in the cytoplasm (Fig. 3B).

The identity of the cytoplasmic TLCV viral nucleic acid was also investigated by strand-specific probing. TLCV-infected sections probed with CS-rep, designed to detect viral DNA but not transcripts (Fig. 1A; Table 1), exhibited chromogenic signals in both the nuclei and cytoplasm of infected cells (Fig. 3G). This observation confirmed the results of the RNase treatment (Figs. 3A and D). As a control, a TGMV specific CS-rep probe was hybridized to TGMV-infected tissues. Strong signals were observed only in the nuclei of TGMV-infected cells (Fig. 3H), consistent with results following the RNase treatment (Fig. 3E).

We extended our study of subcellular DNA accumulation to the monopartite *Tomato yellow leaf curl Sardinia virus* (TYLCSV). The number of infected cells in sections obtained from TYLCSV-infected *N. benthamiana* plants was considerably less than in sections from TLCV-infected plants, and chromogenic signals in TYLCSV-infected plants were always weaker (compare Figs. 1C4, 2A, and 3A with Fig. 4A). Using a strand-specific probe to detect TYLCSV DNA but not its transcripts (CS-rep; Table 1), chromogenic signals were mainly observed in the nuclei of infected phloem cells. Cytoplasmic signals were also present, but these were relatively weak and limited to a few cells (Fig. 4B). Thus, the localization pattern of TYLCSV DNA in *N. benthamiana* appears to be intermediate between TLCV and TGMV. It is possible that the low level of TYLCSV DNA signals reflects the reduced titer of this virus in *N. benthamiana* plants compared to TLCV. However, the pattern of TYLCSV DNA accumulation suggests that cytoplasmic localization is not unique to TLCV.

Cytoplasmic TLCV signals are essentially due to ssDNA

To determine whether cytoplasmic TLCV DNA was ss or ds, tissue sections were hybridized with TLCV-specific virion- and complementary-sense probes homologous to a part of the intergenic region (IR) (Fig. 1A) which is not transcribed to a detectable level (Dry et al., 1993). CS-ir probe, designed to detect both ss- and dsDNA forms (Fig. 1B and Table 1), produced strong chromogenic signals in the cytoplasm (Fig. 5B). The VS-ir probe, designed to target dsDNA only (Fig. 1B and Table 1), produced strong signals only in the nuclei (Fig. 5A) after a denaturation step (see Materials and methods). This observation confirmed that dsDNA only contributed to signals from the nucleus and suggested that cytoplasmic signals were due to ssDNA.

To further test this hypothesis, the target DNA in tissue sections was denatured by heat denaturation prior to hybridization (see Materials and methods) with either CS-cp (Fig. 5F) or

Fig. 1. Specificity of the RNA probes. (A) Genome organization of *Tomato leaf curl virus* (TLCV) and *Tomato golden mosaic virus* component A (TGMV A) showing the positions and orientations of strand-specific RNA probes (thin arrows). Thick arrows show the viral ORFs. Target nucleic acids for each probe are listed in Table 1. (B) Demonstration by gel blot analysis of total nucleic acid extracts of *N. benthamiana* and tomato agroinoculated with either TLCV or TGMV that digoxigenin (DIG)-labeled probes shown in panel A specifically target viral DNA. The positions of the open-circular (OC), linear (Lin), supercoiled (SC), and single-stranded (ss) DNA forms characteristic of geminivirus DNA replication, and subgenomic (SG) DNAs, are shown. I = infected, H = healthy. (C) Transverse sections derived from *N. benthamiana* leaf veins were used for hybridization with viral specific probes. The probes and sections used are indicated. The blue color (indicated by arrows) in C4 and C5 signifies the presence of target nucleic acids. PP = phloem parenchyma, XP = xylem parenchyma, X = xylem, CC = companion cells, SE = sieve elements, BS = bundle sheath, M = mesophyll, VC = vascular cambium. Scale bars = 100 μm.

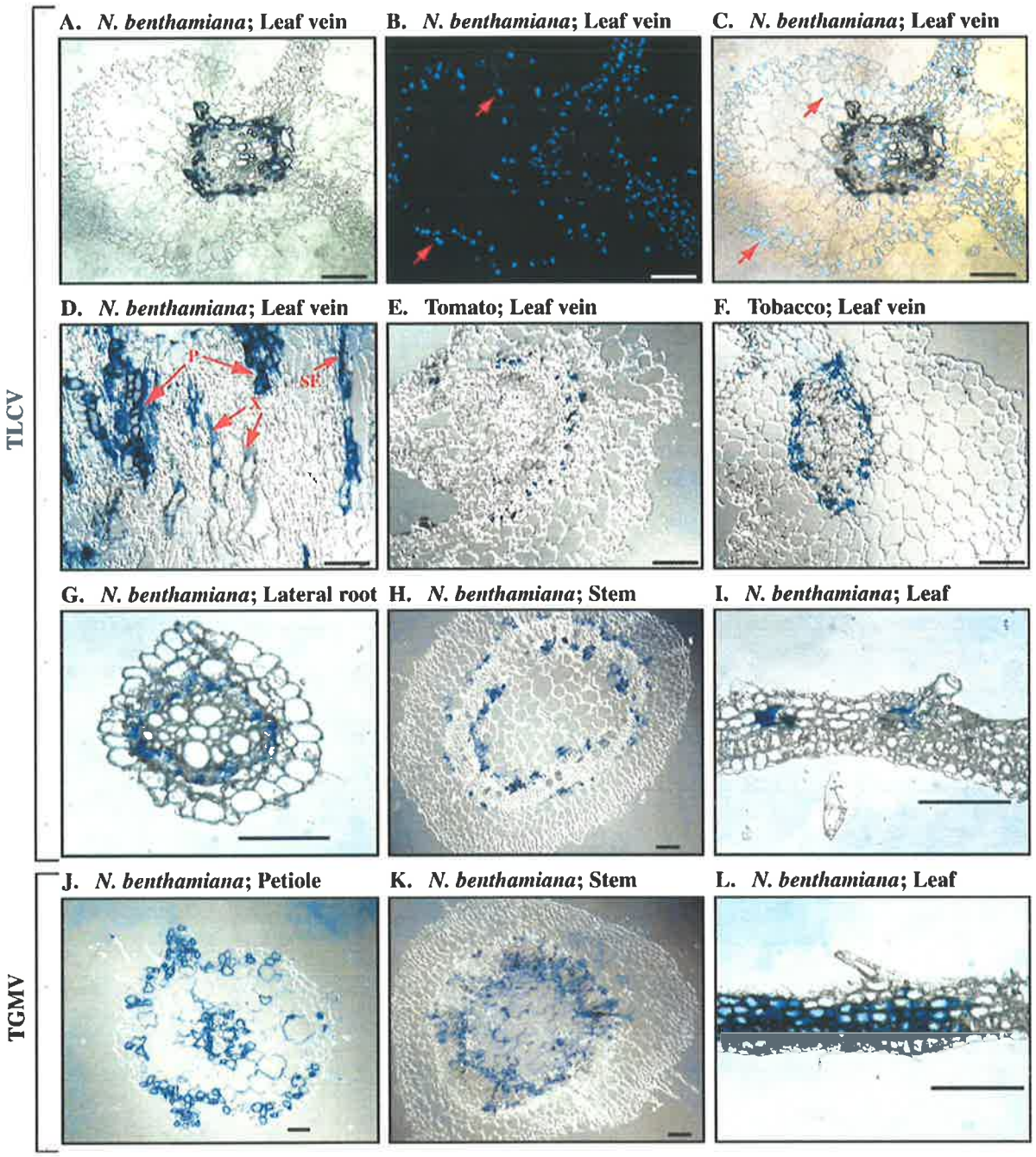


Fig. 2. Comparative in situ localization of TLCV and TGMV. Tissue sections of TLCV-infected *N. benthamiana*, tomato, and tobacco hybridized with TLCV-specific CS-cp DIG probe are compared with TGMV-infected *N. benthamiana* probed with TGMV CS-cp DIG probe. Sections were viewed with differential interference contrast (DIC) optics (D–L) or with bright-field illuminator (A). Nuclei (red arrows) were identified by staining with DAPI (B). Panel C is a merged image of (A) and (B). Tissue type, host plant, and viral infection are indicated. All sections were transverse, except panel D which was longitudinal. P = phloem, X = xylem, SE = sieve elements. Scale bars = 100 μm.

CS-rep probes (data not shown) and its chromogenicity compared with non-denatured sections. No difference in signal intensity was found between sections hybridized following denaturing (Fig. 5F) and non-denaturing treatments (Fig. 5C), indicating that TLCV ssDNA, not dsDNA, accumulates in the cytoplasm. Analysis of the DNA forms present in TLCV-infected plants by two-dimensional agarose gel electrophoresis has shown that ssDNA is the predominant form of viral DNA (Alberter et al., 2005). We also observed a higher level of ssDNA compared to dsDNA (Fig. 1B). Nevertheless, signal intensity was stronger in denatured sections (Fig. 5E) than non-denatured ones (Fig. 5D) using a virion-sense probe that can detect viral dsDNA (VS-cp: Table 1), indicating that a proportion of nuclear signal represents dsDNA form. Faint

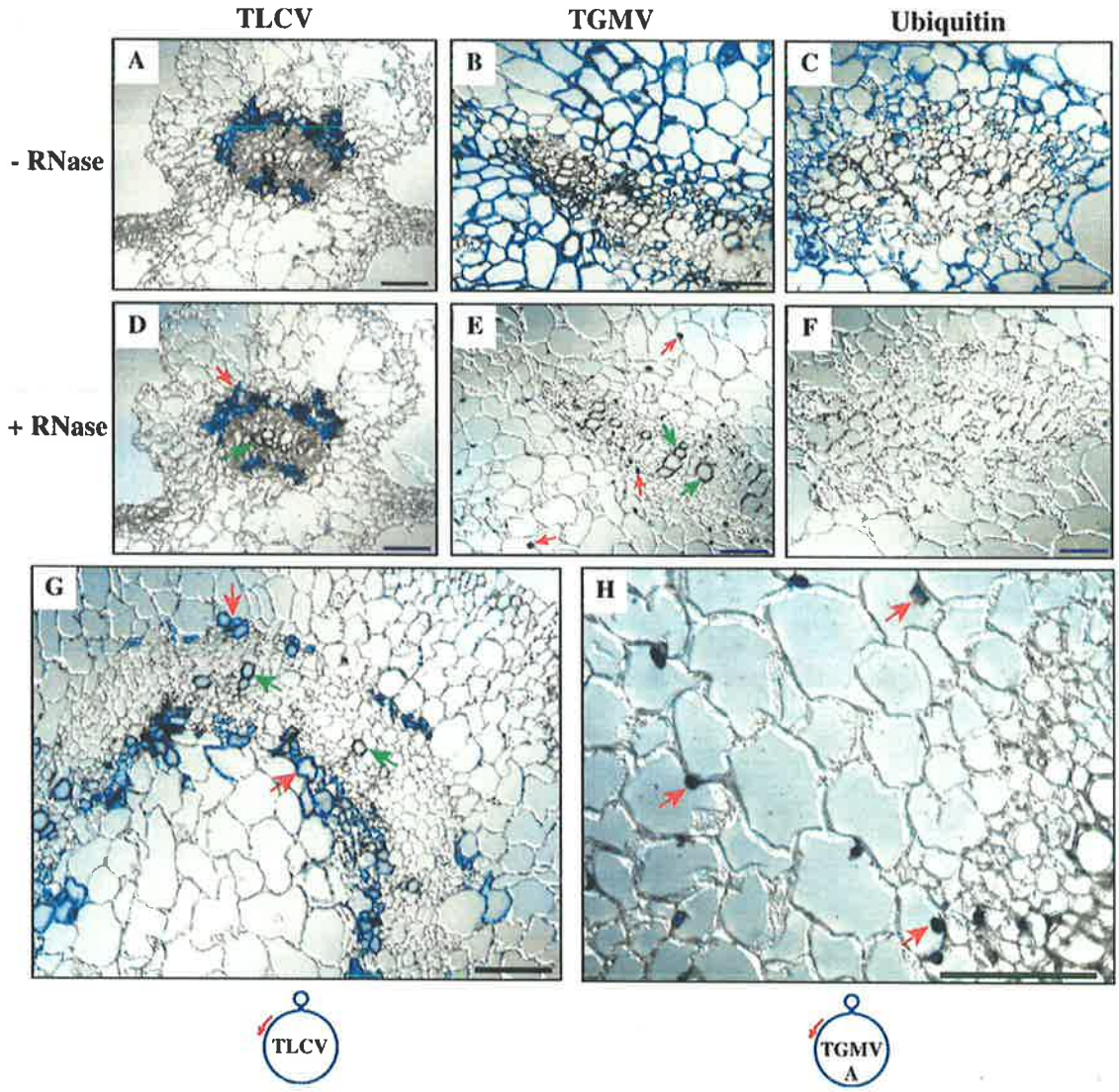


Fig. 3. Differentiating viral DNA from transcripts. Transverse sections of leaf vein (A–F) and stem (G–H) of *N. benthamiana* showing hybridization of a TLCV-specific CS-cp probe with TLCV-infected sections (A, D) and a TGMV-specific CS-cp probe with TGMV-infected sections (B and E). The control was complementary-sense probe for ubiquitin gene transcripts hybridized with non-infected sections (C and F). −RNase, no enzyme treatment; +RNase, treatment with DNase-free RNase. Transverse sections of either TLCV-infected (G) or TGMV-infected (H) tissues were hybridized with CS-rep DIG-labeled probes. Red arrows indicate the nuclei containing TGMV DNA (E and H) and TLCV ssDNA (D and G) in phloem cells, and green arrows (D, E, and G) indicate infected xylem. Scale bars = 100 μm.

staining signals, as seen in Fig. 5D, are probably due to the presence of cs ssDNA (see also Fig. 1B, lanes 1 and 5) known to be associated with geminivirus infection (Saunders et al., 1991). Together, these results indicate that TLCV ssDNA accumulates both in the cytoplasm and the nucleus, while dsDNA is present in the nucleus only.

ssDNA of TLCV, TYLCV, and TGMV accumulates in xylem

Xylem vessels are inert tubes which are involved in water transport and provide mechanical support to plant tissues (Boyce et al., 2004). It is generally assumed that plant viruses move systemically through phloem, although some RNA viruses, including sobemoviruses (Moreno et al., 2004; Opalka et al., 1998; Schneider and Worley, 1959), a potyvirus (Dicenta et al., 2003), and a furovirus (Verchot et al., 2001) have been found in xylem. As yet, no DNA virus has been reported to localize to xylem vessels. Using probes specifically designed to detect ssDNA (Table 1; Fig. 1A), we observed the ssDNA of three geminiviruses {TLCV (Figs. 2A, D, 3D and G; see also Fig. 7), TGMV (Figs. 2J, K, 3E, 4C and D) and TYLCSV (Figs. 4A and B)} in xylem vessels. It is important to note that no TLCV (Figs. 5A, D, and E), TGMV, and TYLCSV (data not shown) dsDNA was observed in the xylem.

Viral DNA was detected more frequently in the xylem of younger leaf veins than in stem sections (Table 2) taken immediately beneath the younger leaf (compare Figs. 3D with G

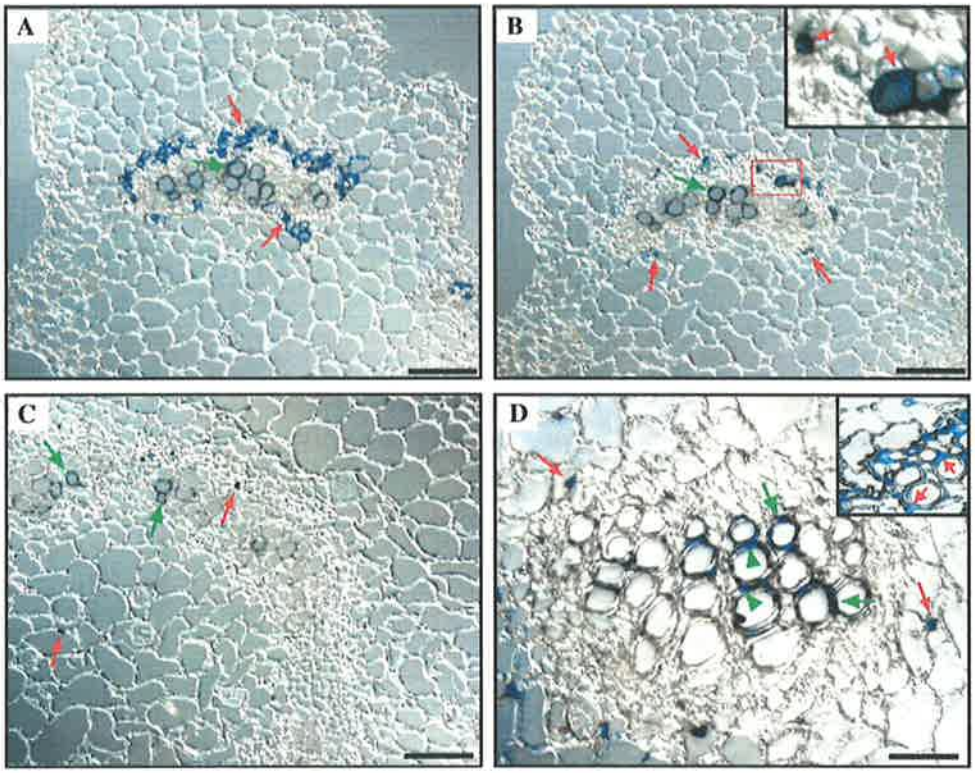


Fig. 4. Localization of TYLCSV and TGMV in xylem. Localization of TYLCSV (A and B). Transverse sections of leaf vein from TYLCSV-infected *N. benthamiana* hybridized with TYLCSV CS-cp (A) or CS-rep (B) probes. The area inside the red box is enlarged in the inset in panel B. Localization of TGMV in xylem (C and D). Transverse sections of stem (C) and leaf vein (D) from TGMV-infected *N. benthamiana* hybridized with TGMV CS-rep probe. Red arrows show the viral-specific hybridization signals in infected cells, and green arrows indicate the viral-specific signals in xylem. Arrow heads in panel D show the spots of stronger hybridization signals indicative of xylem pits. Inset in panel D is the image of vascular cells stained with DAPI showing nuclei of immature xylem (red arrows) located next to developed xylem. Scale bars = 100 μm.

and 4D with C). Viral DNA was not detected in the xylem vessels of mature leaves (data not shown). Unlike other cell types where TGMV ssDNA accumulates in the nucleus (Fig. 4D; red arrows), xylem vessels exhibited TGMV ssDNA signals in the entire vessel (Fig. 4D; green arrows). This is because a mature xylem vessel does not contain nuclear and cellular contents (Fukuda, 1997). Small spots of high chromogenic intensity were also evident in some xylem vessels (green arrowheads; Fig. 4D), which probably represent the pits of xylem (see Fig. 7e) where more viral DNA accumulates.

Hybridization signals in xylem were shown to be virus specific by the negative results obtained with mock-inoculated plants (Fig. 1C1) and with sections taken from virus infected plants incubated either without probes (data not shown) or with probes not specific to the inoculated virus (Figs. 1C2 and C3). Xylem localization data were further supported by the lack of hybridization signals in xylem vessels using probes designed to exclusively detect viral dsDNA (Figs. 5A and E) or ubiquitin (Figs. 3C and F).

Vascular restriction of TLCV is released by TGMV

The tissue tropism of a geminivirus can change in a mixed infection with another geminivirus. For example, the phloem-limited BGMV was shown to infect mesophyll cells in a mixed infection with TGMV (Morra and Petty, 2000). In TLCV and TGMV co-infected plants, TLCV was mainly associated with vascular cells but could also infect a small number of mesophyll cells (Fig. 6B). To detect both viruses in the same section, CS-rep probes of TLCV and TGMV labeled with DIG and fluorescein respectively were applied simultaneously and detected sequentially (Jowett, 2001). TGMV (red fluorescence) was found in the nuclei of vascular and mesophyll cells, while TLCV (blue precipitate) was restricted mainly to vascular cells and was present in the cytoplasm and nuclei of infected cells (Figs. 6A and B). Examination of 12 tissue sections derived from stems, petioles, and leaf veins of TLCV and TGMV co-infected plants identified 3212 TGMV-infected cells but only 855 TLCV-infected cells, of which 726 (~85%) were vascular-associated and the remaining 128 (~15%) were in the mesophyll. The majority of TLCV-infected cells (95%) also contained TGMV. However, a few of TLCV-infected cells did not show TGMV DNA. It is not clear whether these cells did not contain TGMA DNA or the red signals of TGMV DNA were completely masked by the strong blue signals produced by TLCV probe. Alternatively, the lack of TGMV DNA in those cells could be due to the absence of nuclei in the sections. We also noted that the number of cells in which TGMV was detected depended on the probe used. A TGMV-specific probe, which could detect both viral DNA and transcripts, was able to

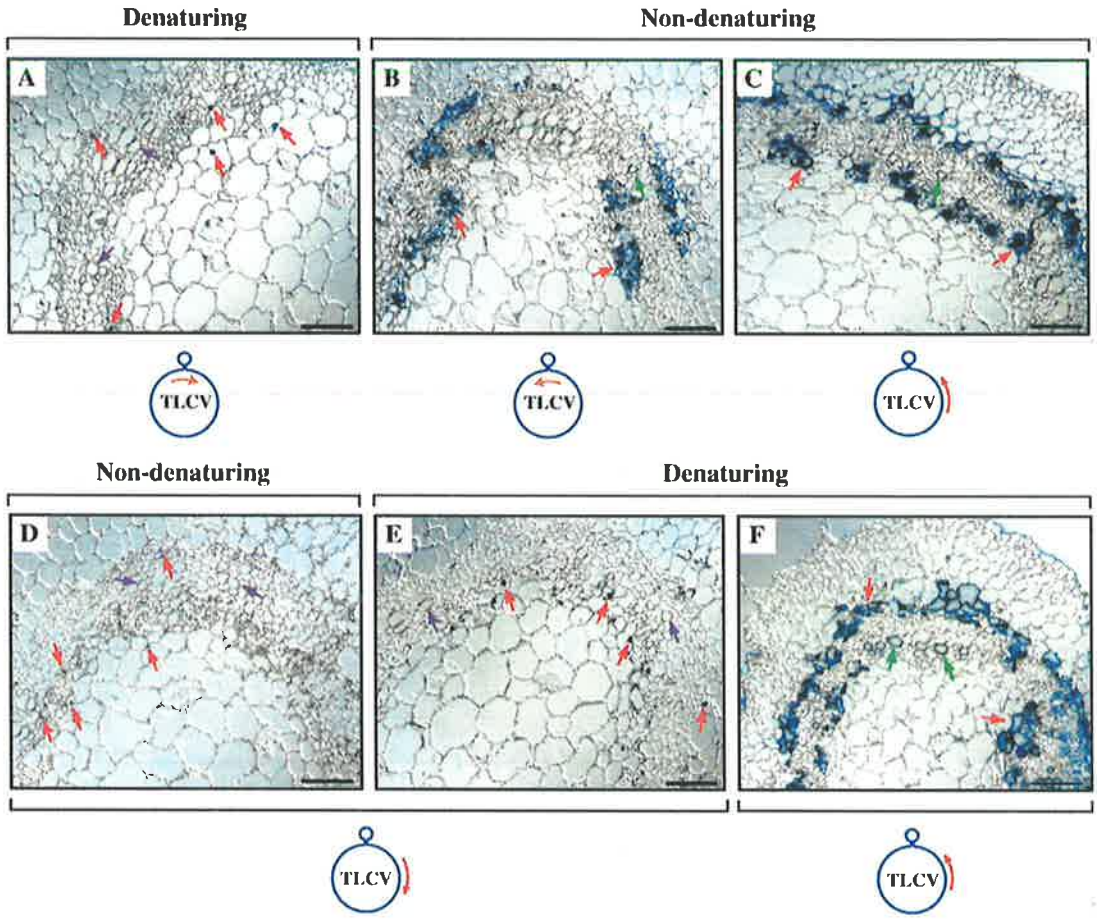


Fig. 5. Differentiating ssDNA from dsDNA. Transverse sections of stem from TLCV-infected *N. benthamiana* hybridized with the probes indicated after denaturing and non-denaturing treatments as described in the methods. Red arrows indicate the nuclei containing the complementary-sense ssDNA (D) and the replicative form of TLCV dsDNA (A and E), red arrows (B, C, and F) indicate TLCV ssDNA in phloem cells, green arrows (B, C and F) indicate infected xylem and purple arrows (A, D, and E) indicate xylem vessels lacking viral signals. Scale bars = 100 μm.

identify many infected cells (Figs. 1C5 and 2J–L), whereas a TGMV-specific probe that could only hybridize to viral DNA detected a small number of infected cells (Figs. 6A and B). As explained earlier, this is probably the result of nuclei not being present in the cell sections due to their relatively smaller size. These results indicate that TGMV can partially alleviate phloem restriction of TLCV.

Discussion

Cell-to-cell movement of geminiviruses involves replication in nuclei, nuclear export of viral progeny, and movement of virus through the cytoplasm to plasmodesmata (Gafni and Bernard, 2002). Previously, geminiviral DNA has been detected in the nuclei of infected cells (Morilla et al., 2004; Morra and Petty, 2000; Qin and Petty, 2001; Qin et al., 1998; Wege et al., 2001). Consequently, DNA movement through the cytoplasm has been considered to be transient (Zhang et al., 2001). Here, we report that TLCV ssDNA accumulates in the cytoplasm at significant levels, while replicative dsDNA is present in the nucleus. However, consistent with earlier findings, we were unable to detect cytoplasmic viral DNA in TGMV infections. Our results suggest that these viruses may utilize different modes of DNA transport across cytoplasm.

Three independent experiments, RNase digestion, strand-specific probing, and hybridization under denaturing and non-denaturing conditions, confirmed that TLCV ssDNA accumulates in the cytoplasm. Morilla et al. (2004) have recently reported that TYLCSV DNA localized predominantly in the nucleus. Consistent with this, we found that ssDNA of the phloem-specific, monopartite TYLCSV accumulates mainly in the nucleus, although we did observe some viral ssDNA in the cytoplasm. It is possible that this discrepancy simply reflects the different in situ hybridization methods used in these

Table 2
Localization of TLCV, TGMV, and TYLCSV in xylem vessels

		TLCV	TYLCSV	TGMV
Leaf[a]	H	43 (59.5%)	45 (61.5%)	56 (59%)
	I	30 (40.5%)	34 (38.5%)	41 (41%)
Stem	H	336 (87%)	332 (90.5%)	321 (88%)
	I	51 (13%)	35 (9.5%)	44 (12%)

[a] Five sections each derived from leaf midrib or stem were observed and healthy (H) or infected (I) xylem vessels were counted.

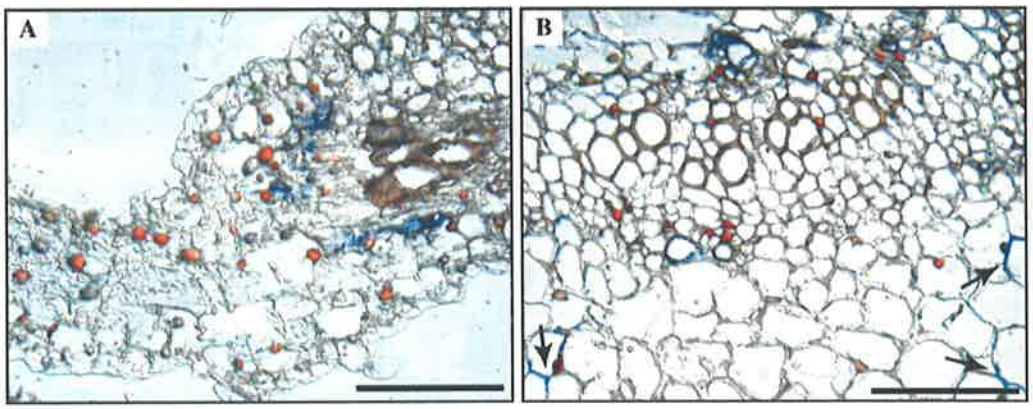


Fig. 6. TLCV presence in mesophyll tissues co-infected with TGMV. Transverse sections from leaf lamina (A) and stem (B) taken from *N. benthamiana* plants co-infected with TLCV and TGMV were hybridized simultaneously with TLCV CS-rep DIG-labeled probe (blue) and TGMV Cs-rep fluorescence-labeled probe (red) and stained sequentially. Non-vascular cells infected by TLCV are indicated by arrows (B). Scale bars = 100 μm.

studies. In our work, we used strand-specific probing to differentiate viral DNA forms. Moreover, a proteinase K treatment was carried out in all in situ hybridization experiments. Such a treatment may allow detection of viral ssDNA complexed with CP (Palanichelvam et al., 1998) which acts as an ssDNA binding protein in the cytoplasm. Supporting this idea is the observation that the CP of TYLCV is detectable in the cytoplasm of infected cells using immunolocalization, while a CP:GFP fusion protein expressed in the absence of other viral proteins localizes to the nucleus (Rojas et al., 2001). The cytoplasmic TLCV ssDNA may be encapsidated or present as a nucleoprotein complex with CP or with host ssDNA binding proteins. The significance of TLCV ssDNA accumulation in the cytoplasm and its possible role in viral movement is presently unknown.

It remains unclear whether ssDNA or dsDNA is involved in viral trafficking. Studies with bipartite begomoviruses are contradictory, supporting the involvement of ssDNA (Padidam et al., 1999; Pascal et al., 1994), dsDNA (Rojas et al., 1998), or both ss- and dsDNA (Hehnle et al., 2004). Based on our finding that ssDNA of TLCV accumulates in the cytoplasm, and the presence of ssDNA of the three viruses studied here in the xylem vessels (discussed below), we suggest that ssDNA may be involved in the movement of these viruses.

It has been suggested that viral, host, and environmental factors (Ding et al., 1999; Kong et al., 2000; Morra and Petty, 2000; Wang et al., 1996) determine the tissue tropism of geminiviruses. Our results indicate that TLCV is restricted to the vascular and bundle sheath cells of tissues derived from leaf, stem, and root of different host plants at different developmental stages and inoculation period. This suggests that vascular limitation of TLCV is mainly determined by viral factors and is consistent with the theory that phloem-limited viruses may lack cell-to-cell movement functions in non-vascular tissues (Taliansky and Barker, 1999). When co-infected with the mesophyll-invasive TGMV, we found that the vascular limitation of TLCV was partially abrogated. The mechanism by which TGMV can release phloem restriction of TLCV remains unclear.

In this study, we observed the presence of geminiviral ssDNA in xylem tissue. A model depicting the route of viral entry to xylem cells is shown in Fig. 7. Xylem arises from the vascular cambium through transdifferentiation (Fukuda, 1997). Immature xylem cells contain active nuclei (Mauseth, 1988). Such immature cells are evident in inset to Fig. 4D. Subsequently, these cells undergo programmed cell death and lose their nuclei and cell contents, leaving hollow dead cells that form vessels or tracheids (Fukuda, 1997). We suspect that geminiviruses preferentially localize to vascular cambium, comprising the most actively dividing cells in the vasculature (Mauseth, 1988), and then spread to phloem and xylem parenchyma by two separate processes: actively, by cell-to-cell movement, and passively, through the differentiation of cambium cells. Several lines of evidence support this hypothesis. First, TLCV DNA was not observed in mature leaves where cambium cells are not present (Mauseth, 1988). Second, a larger proportion of xylem vessels were infected in young leaves, where transdifferentiation from cambium cells is highly active, than in stem tissue, where this process is largely absent. Third, the localization of geminiviruses to xylem cells is supported by an increasing body of evidence implicating xylem tissue in long-distance transport of plant viruses (Dicenta et al., 2003; Moreno et al., 2004; Opalka et al., 1998; Schneider and Worley, 1959; Verchot et al., 2001). Opalka et al. (1998) proposed a model to explain the systemic movement of virus from apoplast to symplast whereby *Rice yellow mottle virus* (RYMV) chelates calcium in the pit membrane of xylem to destabilize the membrane structure for its movement to living immature tracheids.

We have considered whether the viral DNA signal observed in xylem tissue is due to the agroinoculation procedure, where *Agrobacterium* containing viral DNA could be directly introduced to xylem cells. However, we are not aware of any reports indicating movement of *Agrobacterium* through xylem. Furthermore, the observed localization of viral DNA preferentially in young leaves compared to old leaves, which are closer to the site of inoculation, indicates that this is highly unlikely.

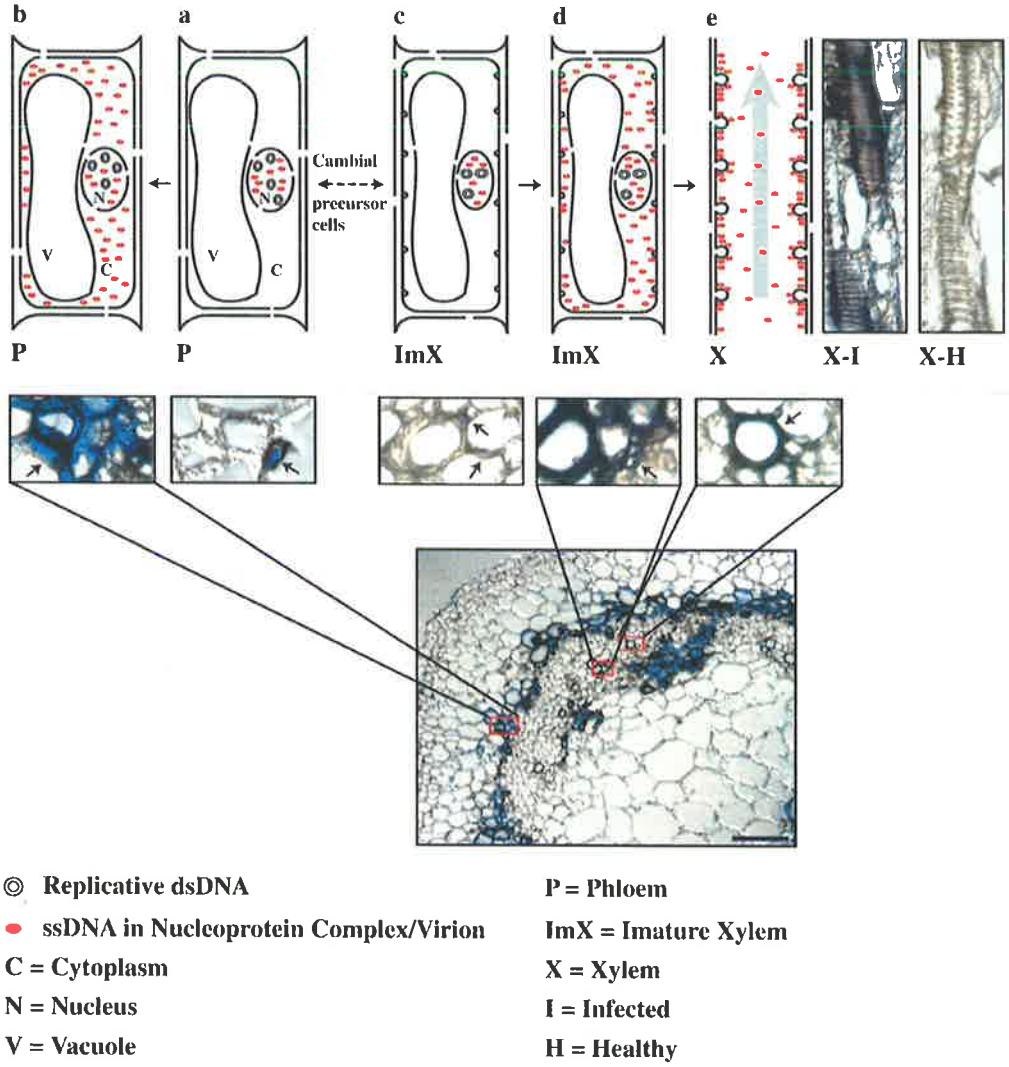


Fig. 7. A model derived from the in situ localization studies to explain the distribution of TLCV ss- and dsDNA in the stem vasculature of *N. benthamiana*. (a) A replicative form of viral DNA (dsDNA) is present in the nucleus of phloem cells (P). Once a significant amount of ssDNA synthesized in the nucleus, then it accumulates in the cytoplasm (b). During differentiation (c–e) of immature xylem cells (ImX) to xylem (X) through programmed cell death, which results in the loss of cellular contents, viral ssDNA either in the form of nucleoprotein complex or virion particles accumulates in the mature xylem vessels especially around the pits of xylem (e). Longitudinal sections of infected xylem (X–I) and healthy xylem (X–H) are shown. Lower panels show the viral forms explained in the model hybridized with the probes specific to detect only dsDNA or both ss- and dsDNA as explained in the text.

Our finding that TLCV ssDNA accumulates in the cytoplasm may represent a new feature of the lifecycle of some geminiviruses. Moreover, the differential DNA accumulation pattern of TLCV and TGMV is suggestive of disparate movement mechanisms of monopartite and bipartite viruses.

Table 3
Infectious clones of TLCV, TGMV, and TYLCSV DNA

Plasmid designation	Construct	Source
pBin TLCV	2.0 mer	Dry et al. (1993)
pBin TGMV A	2.0 mer	von Arnim and Stanley (1992)
pBin TGMV B	2.0 mer	von Arnim and Stanley (1992)
pBin TYLCSV	2.0 mer	Kheyr-Pour et al. (1991)

Materials and methods

Plant material and inoculation

N. benthamiana, tobacco (*N. tabacum* cv. Samsun), and tomato (*S. lycopersicum* L. cv. Grosse Lisse) were maintained at 25–30 °C with a 16-h photoperiod under containment conditions. Plants at the 4–5 leaf stage were inoculated with infectious viral clones (Table 3) using *Agrobacterium tumefaciens* (Grimsley et al., 1987). Young (3rd), developing (7th), and mature (12th) leaves, stems, petioles, and roots were sampled at the 3–4 weeks post-inoculation and processed for fixation as described below. Infection was monitored by dot blot analysis of viral DNA using ^{32}P-labeled complementary DNA probes (Stonor et al., 2003).

Tissue processing

Tissues were cut into 4 mm by 6 mm pieces and fixed in 4% paraformaldehyde: 0.25% glutaraldehyde and were embedded in buty-methyl methacrylate (Nadeau et al., 1996). Sections of 8 μm in thickness were obtained with a rotary microtome (Model 2055; Leica, Germany) and placed on silane-coated slides (ProSciTech, Thuringowa Central, Australia).

Preparation of RNA probes

DNA fragments were amplified by the polymerase chain reaction (PCR) using specific primers (Table 4). The amplified DNA was ligated into pGEM-T-Easy (Promega, Madison, WI), and orientation of each insert was checked by sequencing. Plasmids were linearized with appropriate restriction enzymes and transcribed with T7 DNA-dependent RNA polymerase (Promega). Probes were labeled with either digoxigenin (DIG)-11-dUTP or fluorescein-12-dUTP using DIG or fluorescein RNA Labelling Mixes (Roche Diagnostics, Castle Hill, Australia). After transcription, DNA templates were removed by digestion with RNase-free DNase I, and the probes were hydrolyzed to approximately 150 nt by carbonate treatment (Guerin et al., 2000).

DNA gel blot analysis using non-radioactive probes

Total nucleic acid was extracted as described previously (Dry et al., 1993) from TLCV and TGMV infected *N. benthamiana* and tomato plants. The DNA was electrophoresed in a 1.2% agarose gel, blotted onto Zeta-Probe membrane (BioRad, Hercules, CA) (Dry et al., 1993), and hybridized overnight at 42 °C with strand-specific DIG-labeled TLCV- and TGMV-specific probes (Fig. 1A; Table 3). Membranes were washed with 2× SSC (1× SSC = 0. 15 M NaCl and 0.015 M trisodium citrate) twice for 5 min each and then once for 30 min with 0.2× SSC containing 0.3% Triton-X100. The membranes were blocked in 1× blocking solution (Roche Diagnostics) for 30 min, soaked in TST buffer (100 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.3% Tween 20) containing 1% bovine albumin serum (BSA) for 30 min, incubated in 1% BSA TST buffer containing anti-DIG alkaline phosphatase (AP; 1:5000) for 30 min, washed twice in TST buffer for 10 min each, and then equilibrated in detection buffer (0.1 M Tris–HCl pH 9.5, 0.1 M NaCl, 0.05 M $MgCl_2$) for 5 min. AP activity was detected by incubating in detection buffer containing 4.5 μl ml^{-1} nitroblue tetrazolium (NBT; Roche Diagnostics) and 3.5 μl ml^{-1} 5-bromo-4-chloro-3-indolyl-phosphate (BCIP; Roche Diagnostics) for 30–60 min until a dark-bluish color developed. The reaction was stopped with TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 7.5).

Table 4
Oligonucleotide primers

Name	Sequence (5′to 3′)	Description
P1	GG**GAATTC**TGGGATCCTTTAGTCCAC[a]	TLCV V1-F
P2	GGTT**CTCGAG**TCAGGGCTTCTGAACAGC[a]	TLCV V1-R
P3	GG**GAATTC**AGCAAGCGACCAGCAGAT[a]	TLCV V2-F
P4	GG**GGATCC**TTAATTCTGAATCGA[a]	TLCV V2-R
P5	TT**GAATTC**ATGAGAATGGGGAGCCTC[a]	TLCV C4-F
P6	GT**GGATC**CATTCCCTAAGGACGTTA[a]	TLCV C4-R
P7	TCG**GAGCTC**GTGTCTGGGGTCTTAT[a]	TLCV IR-F
P8	GGGCCCAAGTATATATACGACAAAAAAC	TLCV IR-R
P9	GG**CATATG**CAAGATATGGATGGATG[a]	TGMV AV1-F
P10	TCCTAACCAGAGCCTGCTCGTTG	TGMV AV1-R
P11	CCG**CATATG**GCCGCGCAGCGGA[a]	TGMV AC1-F
P12	CTAACGACGCTGCAGCAGAGGCGT	TGMV AC1-R
P13	CCA**CTGCAG**ATGCCGAAGCGAACC	TYLCSV V1-F
P14	CCGTTAATTTGTTACAGCATCATAAAAATAA	TYLCSV V1-R
P15	CCG**CTGCAG**ATGAAAATGGGGAACC[a]	TYLCSV C4-F
P16	CCGTTACATCAAGAGCCTGCGACTTA	TYLCSV C4-R
P17	GGGATGCAGATCTTCGTGAAAACCC	Ubiquitin-F
P18	TCAATCGCCTCCAGCCTTGTTGTAA	Ubiquitin-R

F: Forward Primer; R: Reverse Primer.
[a] Specific restriction sites are shown in bold type.

In situ hybridization

Pre-hybridization, hybridization, and post-hybridization were done as previously described (Guerin et al., 2000). The DIG- or fluorescein-labeled probes were detected by anti-DIG or anti-fluorescein fab alkaline phosphatase conjugates (Roche Diagnostics) and subsequently stained by NBT/BCIP or Fast Red substrates (Roche Diagnostics). Dual color in situ hybridization was done by applying the probes simultaneously and detecting them sequentially (Jowett, 2001). For RNase treatment, the in situ hybridization protocol was modified as follows: the slides were incubated in 1× TE buffer (10 mM Tris–HCl pH 8.0, 1 mM EDTA) containing 20 μg ml^{-1} DNase-free RNase-A for 50 min at 37 °C, followed by two washes in TE buffer. Proteinase treatment was done using 100 mM Tris–HCl (pH 8.0), 50 mM EDTA containing 1 $mg.ml^{-1}$ proteinase K. To inactivate RNase A, slides were placed in PBS buffer (0.13 M NaCl, 0.007 M Na_2HPO_4, 0.003M Na_2HPO_4, pH 7.0) containing 0.1% diethyl pyrocarbonate (DEPC) for 20 min. To hydrolyze DEPC, the slides were washed with PBS and then incubated in 1× NTE (500mM NaCl, 10 mM Tris–HCl pH 8.0, 1 mM EDTA) two times for 15 min each prior to hybridization.

To differentiate ssDNA from dsDNA, in situ hybridizations were done under both denaturing and non-denaturing conditions. To denature target DNA in tissue sections, slides covered with formamide hybridization buffer containing probe mixture were placed on a hot plate at 80 °C for 4 min prior to hybridization at 42 °C. Non-denaturing hybridization was performed at 42 °C without prior heat treatment of the slides.

Sections were photographed with a SPOT digital camera mounted on a Zeiss Axioplan microscope (Germany) using differential interference contrast (DIC) optics and SPOT software. The contrast and brightness of images were subsequently adjusted and processed using Adobe Photoshop software (Adobe Systems Inc., San Jose, CA).

Acknowledgments

We are grateful to Susan Johnson for the excellent technical assistance with the in situ experiments, Satish Dogra for the helpful discussion, John Stanley for providing the infectious clones of TGMV, Bruno Gronenborn for providing the

infectious clones of TYLCSV, Ghafar Sarvestani for the assistance with the confocal scanning laser microscope, Angela Renfry and Meredith Wallwork for helping with tissue identification. M. S. Rasheed was supported by an ACIAR/ John Allwright Fellowship.

References

Alberter, B., Rezaian, M.A., Jeske, H., 2005. Replicative intermediates of Tomato leaf curl virus and its satellite DNAs. Virology 331, 441–448.

Bass, H.W., Nagar, S., Hanley-Bowdoin, L., Robertson, D., 2000. Chromosome condensation induced by geminivirus infection of mature plant cells. J. Cell Sci. 113, 1149–1160.

Boyce, C.K., Zwieniecki, M.A., Cody, G.D., Jacobsen, C., Wirick, S., Knoll, A.H., Holbrook, N.M., 2004. Evolution of xylem lignification and hydrogel transport regulation. Proc. Natl. Acad. Sci. U.S.A. 101, 17555–17558.

Dicenta, F., Martinez-Gomez, P., Rubio, M., Audergon, J.M., 2003. Localisation and movement of Plum pox virus in apricot stem tissues. Ann. Appl. Biol. 142, 99–105.

Ding, X.S., Flasinski, S., Nelson, R.S., 1999. Infection of barley by brome mosaic virus is restricted predominantly to cells in and associated with veins through a temperature-dependent mechanism. Mol. Plant Microbe. Interact. 12, 615–623.

Dry, I.B., Rigden, J.E., Krake, L.R., Mullineaux, P.M., Rezaian, M.A., 1993. Nucleotide sequence and genome organization of tomato leaf curl geminivirus. J. Gen. Virol. 74, 147–151.

Fukuda, H., 1997. Tracheary element differentiation. Plant Cell 9, 1147–1156.

Gafni, Y., Bernard, L.E., 2002. The role of host and viral proteins in intra- and inter-cellular trafficking of geminiviruses. Physiol. Mol. Plant Pathol. 60, 231–241.

Grimsley, N., Hohn, T., Davies, J.W., Hohn, B., 1987. Agrobacterium-mediated delivery of infectious maize streak virus into maize plants. Nature 325, 177–179.

Guerin, J., Rossel, J.B., Robert, S., Tsuchiya, T., Koltunow, A., 2000. A DEFICIENS homologue is down-regulated during apomictic initiation in ovules of Hieracium. Planta 210, 914–920.

Gutierrez, C., 2000. DNA replication and cell cycle in plants: learning from geminiviruses. EMBO J. 19, 792–799.

Hanley-Bowdoin, L., Settlage, S.B., Orozco, B.M., Nagar, S., Robertson, D., 1999. Geminiviruses: models for plant DNA replication, transcription, and cell cycle regulation. Crit. Rev. Plant Sci. 18, 71–106.

Hehnle, S., Wege, C., Jeske, H., 2004. Interaction of DNA with the movement proteins of geminiviruses revisited. J. Virol. 78, 7698–7706.

Jowett, T., 2001. Double in situ hybridization techniques in zebrafish. Methods 23, 345–358.

Kheyr-Pour, A., Bendahmane, M., Matzeit, V., Accotto, G.P., Crespi, S., Gronenborn, B., 1991. Tomato yellow leaf curl virus from Sardinia is a whitefly-transmitted monopartite geminivirus. Nucleic Acids Res. 19, 6763–6769.

Kong, L.J., Orozco, B.M., Roe, J.L., Nagar, S., Ou, S., Feiler, H.S., Durfee, T., Miller, A.B., Gruissem, W., Robertson, D., Hanley-Bowdoin, L., 2000. A geminivirus replication protein interacts with the retinoblastoma protein through a novel domain to determine symptoms and tissue specificity of infection in plants. EMBO J. 19, 3485–3495.

Maththews, R.E.F., 1993. Diagnosis of Plant Virus Diseases. CRC Press, Boca Raton, FL.

Mauseth, J.D., 1988. Plant Anatomy. Benjamin/Cummings Publication Coperation, California, USA.

Moreno, I.M., Thompson, J.R., Garcia-Arenal, F., 2004. Analysis of the systemic colonization of cucumber plants by Cucumber green mottle mosaic virus. J. Gen. Virol. 85, 749–759.

Morilla, G., Krenz, B., Jeske, H., Bejarano, E.R., Wege, C., 2004. Tete a tete of tomato yellow leaf curl virus and tomato yellow leaf curl sardinia virus in single nuclei. J. Virol. 78, 10715–10723.

Morra, M.R., Petty, I.T., 2000. Tissue specificity of geminivirus infection is genetically determined. Plant Cell 12, 2259–2270.

Nadeau, J.A., Zhang, X.S., Li, J., O'Neill, S.D., 1996. Ovule development: identification of stage-specific and tissue-specific cDNAs. Plant Cell 8, 213–239.

Nagar, S., Pedersen, T.J., Carrick, K.M., Hanley-Bowdoin, L., Robertson, D., 1995. A geminivirus induces expression of a host DNA synthesis protein in terminally differentiated plant cells. Plant Cell 7, 705–719.

Noris, E., Vaira, A.M., Caciagli, P., Masenga, V., Gronenborn, B., Accotto, G.P., 1998. Amino acids in the capsid protein of tomato yellow leaf curl virus that are crucial for systemic infection, particle formation, and insect transmission. J. Virol. 72, 10050–10057.

Noueiry, A.O., Lucas, W.J., Gilbertson, R.L., 1994. Two proteins of a plant DNA virus coordinate nuclear and plasmodesmal transport. Cell 76, 925–932.

Opalka, N., Brugidou, C., Bonneau, C., Nicole, M., Beachy, R.N., Yeager, M., Fauquet, C., 1998. Movement of rice yellow mottle virus between xylem cells through pit membranes. Proc. Natl. Acad. Sci. U.S.A. 95, 3323–3328.

Padidam, M., Beachy, R.N., Fauquet, C.M., 1999. A phage single-stranded DNA (ssDNA) binding protein complements ssDNA accumulation of a geminivirus and interferes with viral movement. J. Virol. 73, 1609–1616.

Palanichelvam, K., Kunik, T., Citovsky, V., Gafni, Y., 1998. The capsid protein of tomato yellow leaf curl virus binds cooperatively to single-stranded DNA. J. Gen. Virol. 79, 2829–2833.

Pascal, E., Sanderfoot, A.A., Ward, B.M., Medville, R., Turgeon, R., Lazarowitz, S.G., 1994. The geminivirus BR1 movement protein binds single-stranded DNA and localizes to the cell nucleus. Plant Cell 6, 995–1006.

Qin, Y., Petty, I.T., 2001. Genetic analysis of bipartite geminivirus tissue tropism. Virology 291, 311–323.

Qin, S., Ward, B.M., Lazarowitz, S.G., Qin, S.W., 1998. The bipartite geminivirus coat protein aids BR1 function in viral movement by affecting the accumulation of viral single-stranded DNA. J. Virol. 72, 9247–9256.

Rezaian, M.A., Krake, L.R., Cunying, Q., Hazzalin, C.A., 1991. Detection of virus-associated dsRNA from leafroll infected grapevines. J. Virol. Methods 31, 325–334.

Rigden, J.E., Dry, I.B., Mullineaux, P.M., Rezaian, M., 1993. Mutagenesis of the virion-sense open reading frames of tomato leaf curl geminivirus. Virology 193, 1001–1005.

Rojas, M.R., Noueiry, A.O., Lucas, W.J., Gilbertson, R.L., 1998. Bean dwarf mosaic geminivirus movement proteins recognize DNA in a form- and size-specific manner. Cell 95, 105–113.

Rojas, M.R., Jiang, H., Salati, R., Xoconostle-Cazares, B., Sudarshana, M.R., Lucas, W.J., Gilbertson, R.L., 2001. Functional analysis of proteins involved in movement of the monopartite begomovirus, Tomato yellow leaf curl virus. Virology 291, 110–125.

Rushing, A.E., Sunter, G., Gardiner, W.E., Dute, R.R., Bisaro, D.M., 1987. Ultrastructural aspects of tomato golden mosaic virus infection in tobacco. Phytopathology 77, 1231–1236.

Saunders, K., Lucy, A., Stanley, J., 1991. DNA forms of the geminivirus African cassava mosaic virus consistent with a rolling circle mechanism of replication. Nucleic Acids Res. 19, 2325–2330.

Schneider, I.R., Worley, J.F., 1959. Rapid entry of infectious particles of southern bean mosaic virus into living cells following transport of the particles in the water stream. Virology 8, 243–249.

Stanley, J., Bisaro, D.M., Briddon, R.W., Brown, J.K., Fauquet, C.M., Harrison, B.D., Rybicki, E.P., Stenger, D.C., 2005. Geminiviridae. In: Fauquet, C.M., Mayo, M.A., Maniloff, J., Desselberger, U., Ball, L.A. (Eds.), Virus Taxonomy: VIIIth Report of the International Committee on Taxonomy of Viruses. Elsevier/Academic Press, London.

Stonor, J., Hart, P., Gunther, M., DeBarro, P., Rezaian, M.A., 2003. Tomato leaf curl geminivirus in Australia: occurrence, detection, sequence diversity and host range. Plant Pathol. 52 (3), 379–388.

Taliansky, M., Barker, H., 1999. Movement of luteoviruses in infected plants. In: Smith, H.G., Barker, H. (Eds.), The Luteoviridae. CAB International, Wallingford, UK, pp. 69–81.

Verchot, J., Driskel, B.A., Zhu, Y., Hunger, R.M., Littlefield, L.J., 2001. Evidence that soilborne wheat mosaic virus moves long distance through the xylem in wheat. Protoplasma 218, 57–66.

Voinnet, O., Pinto, Y.M., Baulcombe, D.C., 1999. Suppression of gene silencing: a general strategy used by diverse DNA and RNA viruses of plants. Proc. Natl. Acad. Sci. U.S.A. 96, 14147–14152.

von Arnim, A., Stanley, J., 1992. Determinants of tomato golden mosaic virus symptom development located on DNA B. Virology 186, 286–293.

Wang, H.L., Gilbertson, R.L., Lucas, W.J., 1996. Spatial and temporal distribution of bean dwarf mosaic geminivirus in *Phaseolus vulgaris* and *Nicotiana benthamiana*. Phytopathology 86, 1204–1214.

Waterhouse, P.M., Smith, N.A., Wang, M.B., 1999. Virus resistance and gene silencing: killing the messenger. Trends Plant Sci. 4, 452–457.

Wege, C., Saunders, K., Stanley, J., Jeske, H., 2001. Comparative analysis of tissue tropism of bipartite geminiviruses. J. Phytopathol. 149, 359–368.

Zhang, S.C., Wege, C., Jeske, H., 2001. Movement proteins (BC1 and BV1) of Abutilon mosaic geminivirus are cotransported in and between cells of sink but not of source leaves as detected by green fluorescent protein tagging. Virology 290, 249–260.

Bian, X.-Y., Rasheed, M. S., Seemanpillai, M. J., & Rezaian, M. A. (2006). Analysis of Silencing Escape of Tomato leaf curl virus: An Evaluation of the Role of DNA Methylation. *Molecular Plant-Microbe Interactions, 19*(6), 614-624.

Selth, L. A., Dogra, S. C., Rasheed, M. S., Randles, J. W., & Rezaian, M. A. (2006). Identification and Characterization of a Host Reversibly Glycosylated Peptide that Interacts with the Tomato leaf curl virus V1 Protein. *Plant Molecular Biology, 61*(1), 297-310.