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Genetic studies on tomato golden mosaic virus and beet curly top virus

Hormuzdi, Sheriar Gustad, Ph.D.

The Ohio State University, 1993
GENETIC STUDIES ON TOMATO GOLDEN MOSAIC VIRUS
AND BEET CURLY TOP VIRUS

DISSERTATION
Presented in Partial fulfillment of the Requirements
for the Degree of Doctor of Philosophy in the Graduate School
of the Ohio State University

By
Sheriar Gustad Hormuzdi, M. Sc. (Horticulture)

********

The Ohio State University
1993

Dissertation Committee:
D. M. Bisaro
A. M. Lambowitz
M. T. Muller
F. D. Sack
A. P. Young

Approved by
Advisor
Department of Molecular Genetics
Dedicated to my parents Gustad and Kian, 
and to Shirin, my little sister.
ACKNOWLEDGMENTS

During my stay at OSU, I have had the good fortune to have become well acquainted with many people. They made it possible for me to maintain the necessary rigor and discipline which has culminated in “Genetic analysis......”.

I would like to thank my research advisor Dr. David Bisaro, whose technical assistance, guidance, and help was crucial for the successful completion of my studies. He has had to suffer through the immense task of meeting my deadlines concerning the revisions of this tome, and for that too, I would like to thank him. I also gratefully acknowledge the advice and guidance provided by Drs. Tony Young, Alan Lambowitz, Mark Muller, Fred Sack, and Roy Tassava, and thank the members of my committee for giving me a lot of leeway on scheduling and manuscript requirements.

Jerry, Diane, Matt, Chris, Olivier, Kurt, the Sunday frisbee group, Mehdi (graduating fears still, wishing them still), Jose (Stick with your brushes and finish me that piece), Rocky, Andy, members of the Bisaro Lab (Mauricio, Patricia, Marcos, Drake, Kevin, Kim, Fred; Dr. Garry Sunter in particular), and all my numerous friends in MG and OSBC, thanks for all the help and for the wonderful time. I would like also to make special mention of the generosity of my friends and hosts, Garry and Glen Grumbling, who were kind enough to accept me into their household these last couple of months.

I am most thankful to MaryAnn Martin, a friend whose help and support has always been offered ungrudgingly, Fred (old punks never die, they just fade away) Meyer, who has often been the unfortunate recipient of my frantic calls concerning hardware and software, and Lynn, her love and friendship have been very welcome in these chaotic times.

And finally, I acknowledge my debt and love to my family who are sorely missed. I appreciate their patience and understanding in allowing me to pursue my whims, and I greatly value their love.
VITA

May 15, 1965................................. Born - Bombay, India

1985............................................. B. Sc. (Agriculture)
Konkan Krishi Vidyapeeth
Maharashtra, India

1987............................................. M. Sc. (Horticulture)
Indian Agricultural Research Institute
New Delhi, India

PUBLICATIONS


FIELDS OF STUDY

Major Field: Molecular Genetics

Genetic studies on geminivirus replication and spread determinants.
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INTRODUCTION

Geminiviruses are a group of small DNA-containing plant viruses that infect a number of economically important plants including dicotyledonous species such as tobacco, tomato, cassava, and sugarbeets, and monocotyledonous species such as wheat and maize. These viruses are characterized by their distinctive virion morphology consisting of a double-icosahedral capsid approximately 18×30 nm in size, and by their genome which consists of one or two molecules of covalently closed circular single-stranded DNA (ssDNA) between 2.5 and 3.0 kb in size (for review, see Lazarowitz, 1987; Davies and Stanley, 1989; Bisaro et al, 1990; Lazarowitz, 1992; Mullineaux et al., 1992; Stanley, 1993)

Geminiviruses are typically divided into three subgroups (Matthews, 1991), and a diagram representing the genomes of characteristic members are presented in Figure 1. All geminiviruses replicate through a double-stranded (ds) DNA replicative form (RF), which contains open reading frames (ORFs) in both strands. These ORFs diverge from a region containing a conserved hairpin sequence. However, subgroup I and II geminiviruses have a monopartite genome (i.e. a genome consisting of a single molecule of circular ssDNA), whereas subgroup III geminiviruses usually have a bipartite genome (i.e. a genome consisting of two molecules of circular ssDNA, both of which are required for infectivity). Also, the number and arrangement of ORFs on the genome varies for the different subgroups although it is generally conserved for all members within a subgroup. In addition to differences in genome organization, biological distinctions relating to the insect vector responsible for transmission, the type of host infected, and host range can also be made. Subgroup I viruses are
FIGURE 1. Diagrams of the geminivirus genomes. Shown are the RF forms of subgroups I, II, and III, represented by the South African isolate of maize streak virus (MSV), the Logan isolate of beet curly top virus (BCTV), and the DNA A and DNA B components of tomato golden mosaic virus (TGMV), respectively. Open reading frames (ORFs) located on the genomes are indicated by the arrows. Leftward oriented arrows represent complementary sense ORFs, whereas rightward oriented arrows represent virion sense ORFs. Also indicated are the size of genomes, the molecular weights of the putative proteins in kilo Daltons (kD), and the segment of the genome in the bipartite (CR; the nucleotide sequence in this region is nearly identical in the A and B genome components) and monopartite (IR; intergenic region) geminiviruses wherein lies the conserved stem and loop motif.
leafhopper-transmitted, and with the exception of tobacco yellow dwarf virus (TobYDV), infect monocotyledonous plants. Subgroup II viruses are leafhopper transmitted and infect dicotyledonous plants. Subgroup III viruses also infect dicotyledonous plants but are transmitted by whiteflies. Subgroup I and II viruses have broad host ranges. BCTV is the sole member of subgroup II.

Plant viruses must encode all functions needed for replication in infected cells and for spread from these cells to other uninfected ones. The processes of replication and spread are the two most fundamental requirements for successful infection of an individual plant. This is underscored by the fact that plant resistance mechanisms are targeted against one or both of these processes. Significant advances in our understanding of geminivirus replication and spread requirements have been made in recent years. Replication of geminiviruses is believed to occur by a rolling circle mechanism in the nucleus of the host cell using dsDNA intermediates. Transport of the virus in the host is thought to be mediated by plasmodesmata, which connect adjacent plant cells. Since our understanding of geminivirus biology and the participation of geminivirus gene products in the life cycle of the virus is derived in large part from current knowledge pertaining to movement of viruses in plants, and rolling circle replication (RCR) of ssDNA phages and plasmids, a review of these fields is given below.

**Movement of Plant Viruses**

When inoculated with a virus, usually only a very small number of plant cells become infected. The virus replicates in these initially infected cells and is then transported into adjacent healthy cells. Therefore, the development of a full range of
symptoms in a susceptible host is dependent not only upon efficient replication of the virus but also upon its successful transport within the plant body.

After being introduced into a cell, the virus will invade neighboring cells through a process of "local" or "cell-to-cell" spread. The enlarging infection site will eventually encounter the vascular tissues which offer the virus the opportunity to move rapidly to the more distal tissues of the plant. This latter process, occurring through phloem tissue, is called "long distance" spread and has received less attention experimentally, probably because of the complexity of cell and tissue types involved. However, in terms of symptom severity and the rapidity with which the infection spreads, long distance movement is probably of prime importance. In fact, local spread may only serve the limited purpose of transporting the virus to the vascular tissues, and may become irrelevant afterwards (Maule, 1991). Long distance spread may be of particular importance in geminivirus infections since many of these viruses are phloem limited and are delivered directly to the phloem by insect vectors in natural infections.

Studies on local spread of the virus provide the basis for much of our present understanding of virus spread requirements and mechanisms. Cell-to-cell spread is a discrete process in the sense that virus replication in the primary infected cell and transport of the viral genome into an adjacent healthy cell are two separate events. This concept has allowed the identification and characterization of proteins important for spread of the virus. These proteins are termed 'movement proteins'. The best characterized of these include the movement proteins of the RNA viruses tobacco mosaic virus (TMV), alfalfa mosaic virus (AlMV), and cowpea mosaic virus (CPMV), as well as the movement protein of cauliflower mosaic virus (CaMV), whose genome consists of dsDNA (reviewed by Atabekov et al., 1984; Maule, 1991; Daubert, 1992).
**Plasmodesmata and virus movement.** Movement of plant viruses is a highly complex process involving the participation of movement proteins and a plant vasculature employed in the transport of solutes and macromolecules. Plant cells are enclosed within cellulose walls, thus restricting access of the virus to the adjacent, healthy cell. Also, unlike bacteriophages, plant viruses do not have mechanical adaptations to penetrate these walls. Therefore many plant viruses have evolved spread mechanisms which utilize plasmodesmata. These consist of narrow strands of cytoplasm that penetrate adjoining cell walls to interconnect plant cells, thus forming a community of living protoplasts termed the symplasm (reviewed in Gibbs, 1976; Hull, 1989; Robards and Lucas, 1990; Maule, 1991;).

The physical organization of these channels has almost exclusively been determined by ultrastructural analysis of tissue in the electron microscope. The basic plasmodesmatal structure is a 28 nm canal lined with plasma membrane and containing nine 5 nm particle subunits (Fig. 2). Intercellular transport is believed to occur through permeable spaces, 1.5-3.0 nm in diameter, which are located between the 5 nm subunit structures. A thin strand of modified endoplasmic reticulum, the desmotubule, runs through the center of this channel. The neck region, found at the base of the plasmodesma, may serve as a sphincter which regulates plasmodesmatal permeability. Plasmodesmata represent the most obvious and probably the only route for the cell-to-cell spread of plant viruses even though the upper limit for a molecule to be transported is 800-1000 Daltons, which corresponds to a Stokes radius of 0.7-1.0 nm. The dimensions of a virus particle or even of folded nucleic acid are much larger.

Plasmodesmata are functionally homologous to animal intercellular connections, the gap junctions (reviewed by Lowenstein, 1987; Evans, 1988).
FIGURE 2. Structure of a simple plasmodesma. A. Longitudinal section. B. Transverse section. Figure taken from Citovsky and Zambryski (1991). For a description of the structure refer to the text.
Several pieces of evidence suggest that these two structures may be functionally analogous. Both have a similar basic gating capacity and utilize the Ca2+ agonist IP3 (inositol triphosphate) to mediate their function (reviewed by Robards and Lucas, 1990). Furthermore, proteins from soybean and Arabidopsis thaliana which are antigenically related to a gap junction connexin have been identified (Meiners et al., 1989; Meiners et al., 1991) raising the possibility that gap junctions and plasmodesmata may be composed of similar structural subunits.

Experimental evidence from microscopic, immunochemical and genetic studies directly implicate the plasmodesmata in virus movement (reviewed by Hull, 1989; Maule, 1991; Daubert, 1992). Antibodies raised to putative viral transport protein(s) have been used in immunocytochemical studies and have localized the movement proteins of TMV (Tomenius et al., 1987), CaMV (Linstead et al., 1988), AlfMV (Stussi-Garaud et al., 1987), and CPMV (Wellink et al., 1989) to the cell wall and, more specifically in the case of TMV and CaMV, to the plasmodesmata. Further, in many cases, alterations in plamodesmatal structure and/or permeability have been reported in infected cells. Viruses in the comovirus, nepovirus and caulimovirus groups have been shown to induce cytopathic tubular structures that are continuous with the plasmodesmata (Kim et al., 1971; Jones et al., 1973; Perbal et al., 1993). Similar tubular structures have also been observed for a geminiviruses infection (Kim et al., 1992). Virus particles have been shown to be present in these tubules suggesting that movement of these viruses occurs by the passage of virions through modified plasmodesmata. However, not all viruses are thought to move as virus particles, and in most cases the form in which the virus spreads is not known.

Direct evidence implicating a movement protein in the modification of plasmodesmatal size exclusion limit came from studies on plants transgenic for the 30 kD (P30) movement protein of TMV (Wolf et al., 1989). Whereas plasmodesmata in
control plants had a size exclusion limit of 0.73 nm, the size exclusion limit in transgenic plants expressing P30 was 3.1 nm. However, this increase in plasmodesmatal permeability still cannot account for the transfer of free TMV RNA with molecular dimensions of approximately 10 nm. A hypothesis resolving this apparent incongruity was proposed by Citovsky and Zambryski (1991), based upon their demonstration that the movement proteins of TMV and CaMV are single-stranded nucleic acid binding proteins (Citovsky et al., 1990; Citovsky et al., 1991). They suggested that single-stranded nucleic acid binding may be a common property of plant virus movement proteins and that this binding leads to the formation of an extended protein-nucleic acid complex which facilitates virus movement through the plasmodesmata. This hypothesis is strongly supported by recent evidence indicating that the diameter of P30 complexed with DNA and RNA is 1.5-2.0 nm (Citovsky et al., 1992), which is compatible with the increase in plasmodesmatal permeability reported by Wolf et al. (1989).

Mutagenesis studies have revealed that the TMV P30 protein is multifunctional. Two disparate ss-nucleic acid binding domains in the carboxy-terminal region of the protein (Citovsky et al., 1992) have been identified. Also, a 19 amino acid stretch required for targeting the protein to the plant cell wall (Berna et al., 1991) has been determined, although it is not clear if the residues are required for proper folding of the protein. Interestingly, it was noted that in P30 transgenic tobacco plants, P30 associates primarily with secondary plasmodesmata, which are formed as a leaf matures and the cells elongate (Ding et al., 1992). The significance of this observation has only recently been realized with the discovery that P30 is phosphorylated by a protein kinase activity which is enriched in plant cell wall fractions from secondary plasmodesmata (Citovsky et al., 1993). It has been suggested that phosphorylation may deactivate P30 by sequestering it in plant cell
walls. This may be necessary because the two known biological functions of P30, binding single-strand nucleic acid and increasing plasmodesmatal permeability, may alter normal cellular functions. Other indirect evidence suggests that the movement protein-plasmodesmata interaction may be regulated by phosphorylation. This includes the suggestion that cell-to-cell movement of TMV may be dependent on intracellular levels of cAMP (Atabekov et al., 1990), and the observation that P30 is phosphorylated when expressed in insect cells (Atkins et al., 1991).

Sequence analysis of movement proteins. Transcomplementation experiments, where movement of a virus restricted in a host or tissue type is assisted by a helper virus, suggest that some viruses employ a common function(s) in movement. For example, the phloem-restricted bean golden mosaic geminivirus (BGMV) was shown to invade non-phloem tissue when inoculated along with the legume strain of tobacco mosaic virus (Carr and Kim, 1983). This example also illustrates the fact that the two viruses involved in a transcomplementation experiment may be unrelated. However, transcomplementation is a very specific phenomenon since successful infection is possible only with certain combinations of viruses. Nevertheless, if the majority of viruses code for movement proteins which operate in a similar way, then it may be possible to identify common structural features in the linear amino acid sequences of the movement proteins within and between virus groups. Thus, attempts have been made to relate movement protein sequence similarities with potential biochemical activities or functions.

Refined computer-assisted analysis of movement proteins has detected similarities between movement or transport proteins (Melcher, 1990; Koonin et al., 1991). In one study, Koonin et al. (1991) grouped movement proteins into two families. The larger family included the movement proteins of the tobamo-, tobra-,
caulimo-, and comoviruses; apple chlorotic leaf spot virus; and geminiviruses with bipartite genomes. Despite poor homology, they identified three motifs termed LPL, G, and D, common to members of Family 1, which are also found within members of the family of cellular 90K heat shock proteins (HSP90). These proteins are involved in intracellular trafficking as chaperones (Hendrick et al., 1993). It was suggested that this sequence homology indicates a similar chaperone-like function for the Family 1 movement proteins, which is not inconsistent either with the experimental evidence discussed so far or with current models of plant virus movement (Citovsky and Zambryski, 1991; Deom et al., 1992). The possible involvement of molecular chaperones in movement is intriguing, especially since it has recently been discovered that ORFs capable of encoding HSP70 and HSP90 homologues are located on the genomes of certain ssRNA viruses such as citrus tristeza virus and beet yellows virus (Pappu et al., 1993). Also, it has been suggested that heat shock chaperones may participate in assembling TMV coat proteins into virus-like particles in *Escherichia coli* (Hwang et al., 1993).

The idea that molecular chaperones responsible for protein folding may be involved in processes like movement is conceptually appealing since the manipulation of plasmodesmatal permeability or the movement of a protein-viral genome complex may involve a change in the configuration of the components. However, this hypothesis has yet to be tested experimentally, and therefore the significance of the observations noted above are unclear.

In general, computer studies of this nature have not been successful at detecting strong similarities between movement proteins. It is possible that transport proteins have evolved so as to complement the particular features of the virus life cycle, and so it may be unreasonable to expect striking similarities in their sequences. Furthermore, unlike TMV P30 which is multifunctional, it is possible that the
different biochemical activities required for virus movement may be encoded by
different genes. These factors may contribute to the low level of similarity seen in
computer-assisted analysis of plant virus movement proteins, and for the variable
number of movement genes in plant viruses.

A model for plant virus cell-to-cell movement. A model for plant virus cell-
to-cell movement has been proposed by Citovsky and Zambryski (1991). This model
is mainly based upon genetic and biochemical data obtained with TMV and is
illustrated in Figure 3. A summary of its main features is given below.

- The first step is the formation of the movement protein-nucleic acid complex.
The binding of the movement protein to the nucleic acid results in the formation
of long, thin, unfolded complexes. Functionally, this step is thought to resemble
the binding of unfolded protein precursors by molecular chaperones, which are
involved in assembly of protein complexes as well as in protein transport across
biological membranes.

- The second step is the specific targeting of the transport complexes to
plasmodesmata. Potentially, this step is specified by a 19 amino acid stretch in
P30 which has been demonstrated to be important for localization of the protein to
the cell wall (Gafny et al., 1992). In the transport complex, a large number of
movement protein molecules are cooperatively bound to the transferred nucleic
acid molecule. This complex protects the viral nucleic acid from degradation and
also provides multiple localization signals.

- Third, the movement protein interacts with plasmodesmal regulatory subunits to
increase permeability. By analogy with gap junctions, it is possible that
regulation of plasmodesmal permeability may occur by
phosphorylation/dephosphorylation reactions. If the increase in plasmodesmal
FIGURE 3. Model for the movement of viral nucleic acids across the plasmodesma. Shown in the Figure are the formation of the movement protein-nucleic acid complex and localization of these to the plasmodesma (A), translocation of the complex across the channel (B), and the alternative products of the reaction (C). Figure is adapted from Citovsky and Zambryski, (1991).
permeability of 0.73 to 3.1 nm seen in P30 transgenic plants reflects the biological activity of this protein, then passage of nucleic acid protein complexes with a diameter of 2-3 nm could occur.

• The fourth and final step of virus cell-to-cell movement is the actual translocation of the complex through the channel into the adjacent cell. It is possible that the entire complex may be transported through the channel, or alternatively that movement protein molecules are displaced as the nucleic acid is extruded through the channel.

Whereas certain features of the model have been demonstrated, there are still numerous key questions that need to be resolved. For example, the structural changes that result in alteration of plasmodesmatal permeability, and the participation of host proteins that may affect movement protein function either by regulating its activity or by facilitating its localization to plasmodesmata are as yet unknown.

The model may adequately describe the movement of TMV and, perhaps, a large number of other plant viruses. However, evidence suggests that other movement mechanisms exist. This evidence includes the formation of cytopathic tubules extending to the plasmodesmata by certain viruses, the division of plant virus movement proteins into two families based upon similarity of protein sequence (Koonin et al., 1991), the tissue specificity shown by some viruses, and the demonstration of successful transcomplementation only with specific combinations of viruses. Clearly, virus transport is an extremely complex process requiring further investigation; the studies mentioned above merely outline a single putative mechanism.
A number of viruses and plasmids employ rolling circle replication (RCR) for their multiplication. These include plasmids from gram-positive bacteria such as pC194 (Gros et al., 1989) and mitochondria from yeasts (Maleszka et al., 1991), both of which have a double-stranded DNA genome. Several bacteriophages, including M13 and \( \phi X 174 \), which package their genomes as ssDNA also replicate by RCR (for review see Kornberg and Baker, 1992).

**A general scheme for RCR.** A model for RCR, based on the mechanisms of ssDNA containing coliphages (Kornberg and Baker, 1992), would divide the process into three stages. In the first, genomic ssDNA (+ strand DNA; viral strand DNA) is converted by the host replication machinery to the ds replicative form by synthesis of the complementary strand (SS→RF synthesis). In the second stage, the ds form is amplified by rolling circle replication of the + strand, followed by priming and subsequent synthesis of the complementary strand (RF→RF synthesis). The third stage (RF→SS synthesis) involves asymmetric replication of the + strand by the same rolling circle mechanism employed in RF→RF synthesis, except that complementary strand synthesis is blocked. Genomic DNA then accumulates in large amounts as it is encapsidated into virus particles.

Genomic replication in the M13 and \( \phi X 174 \) families of coliphages follows this general scheme, although the details differ in some significant aspects (Fig. 4). In phage \( \phi X 174 \), phage A protein (gpA) initiates RCR by nicking the ori (origin of replication) site in the + strand of the RF DNA and remains covalently linked to the 5' side of the nick. The free 3' end is then extended by DNA polymerase. The gpA still bound to the 5' end of the displaced strand travels along the replication fork.
+ strand viral DNA, template for SS $\rightarrow$ RF synthesis (carried out by host proteins)

FIGURE 4. Rolling circle replication in M13 and $\phi$X174. The scheme for replication of the genomes of the M13 and $\phi$X174 bacteriophages is illustrated. The RF $\rightarrow$ RF synthesis stage is emphasized. Refer to the text for an explanation of the mechanism and of the functions of the proteins.
facilitating unwinding of the two strands by a DNA helicase, which is the product of the *E. coli* rep gene. This results in the formation of a looped rolling circle. When the replication proceeds to complete the circle and the ori site is regenerated on the progeny + strand, gpA cleaves this site, ligates the two ends of the displaced + strand to generate a circular ssDNA molecule, and is transferred to the progeny RF DNA to initiate a new round of replication. The circularized strand (+ strand) may subsequently be encapsidated or serve as a template for further RF synthesis. The replication mechanisms of filamentous phages such as M13, fd and f1 differ in that the protein responsible for cleaving the + strand at the ori sequence and initiating replication, gene 2 product (gp2), does not form a covalent bond to DNA and does not travel along the template. Thus replication proceeds not via a looped rolling circle but by rolling circles with loose tails.

The switch from RF→RF to RF→SS synthesis. The second phase of RCR results in the formation of enough RF templates to support adequate levels of transcription. Later in the replication cycle, the replication machinery is geared for the synthesis of large amounts of viral ssDNA, which are then encapsidated. The two families of coliphages employ slightly different means of regulating the onset of + strand production. In M13, the relative levels of products of genes 2, 5, and X (gp2, gp5 and gpX) determine whether RF→RF synthesis continues or + strand synthesis takes over. Gp5 is a single-strand DNA binding protein which competes with *E. coli* SSB (single-strand binding) protein for coating the displaced + strand. The viral DNA-gp5 complex constitutes a prepackaging precursor which removes viral DNA from the replication pool. Thus, when gp5 dominates, replication shifts to + strand synthesis. In phage ϕX174 (Aoyama et al., 1986), the switch from RF→RF synthesis to phage assembly with the concomitant accumulation of ssDNA depends upon the
accumulation of gpC and gpJ, and proheads (capsid precursors assembled from gpF, G, H, B, and D in the absence of viral DNA). After nicking and the initiation of unwinding by the gpA protein, gpC, a sequence specific ssDNA binding protein, binds to the complex consisting of the rep protein and gpA covalently attached to ssDNA (see above section for a brief discussion). This binding is competitive with SSB and inhibits + strand synthesis required for RF→RF phase replication. The inhibition of DNA synthesis is relieved in the presence of proheads which subsequently package newly synthesized + strand DNA. GpJ, a non-specific DNA binding protein, associates with the parental RF and is transferred to nascent viral strands, where it condenses the DNA as it is packaged in the virion.

Thus, both viruses employ a specific protein to compete with SSB for binding so that successful competition results in inhibition of complementary strand synthesis and, consequently, of the RF→RF phase of replication. A significant difference in the mechanism employed by the two viruses is that in ϕX174, the switch and phage assembly are co-dependent, since inhibition of leading strand synthesis due to the binding of gpC is relieved only in the presence of capsid precursors. Other relevant differences pertain to the proteins themselves. The gp5 protein is a sequence non-specific ssDNA binding protein which exists in an approximate 1:1300-1600 stoichiometry of gp5 to ssDNA. The gpC protein is a sequence specific ssDNA binding protein and this property is reflected in the cellular ratio of 1 gpC protein per RF DNA molecule. Importantly, cells infected with phages disrupted in the gp5 and gpC genes accumulate RF DNA at the expense of ssDNA. This observation is of some relevance and will be recalled when the phenotypes of BCTV R2 mutants are discussed.
CURRENT STATUS OF STUDIES ON GEMINI VIRUS SPREAD AND REPLICATION

The nucleotide sequences of a number of geminiviruses have been determined. The location and primary sequence of ORFs capable of encoding proteins greater than 10 kD, have been deduced from this nucleotide sequence data. The importance of these ORFs is indicated by the fact that their location and primary sequences are conserved among members of a subgroup (Fig. 1). Genetic and biochemical studies have indicated that the proteins encoded by the ORFs are key components of geminivirus replication and spread mechanisms. The results of these studies will be discussed below and are summarized in Table 1.

Geminivirus movement. For the most part, studies on geminivirus movement have been limited to the identification of genes required for spread. The basic strategy has been to disrupt viral ORFs and examine their replication in transient systems and in planta. Viral DNA of mutants impaired in movement functions will be detected only in the former system, whereas amplification of mutants defective for replication will not occur in either tissue.

Mutagenesis studies of this nature have resulted in the observation that genes R1 and R2 of subgroup I geminiviruses (Lazarowitz 1988; Boulton et al., 1989; Woolston et al., 1989; Boulton et al., 1993), R1 and R3 of subgroup II geminiviruses (Briddon et al., 1989; Stanley et al., 1992a; Hormuzdi and Bisaro, 1993), and BL1, BR1 and AL2 of subgroup III geminiviruses (Rogers et al., 1986; Brough et al., 1988; Elmer et al., 1988a; Etessami et al., 1988; Morris et al., 1988; Sunter et al., 1990; Etessami et al., 1991; Lazarowitz, 1991; Evans et al., 1993) are necessary for efficient movement. Interestingly, the coat protein is required for the spread of subgroup I and II geminiviruses, whereas the bipartite whitefly-transmitted geminiviruses do not
# Table 1

**Summary of Genetic and Biochemical Studies on Geminivirus ORFs**

<table>
<thead>
<tr>
<th>Members&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Genes&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Phenotype of mutant in plants</th>
<th>Phenotype of mutant in protoplasts/leaf discs/inoculation site</th>
<th>Function of gene product</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Subgroup I: Leafhopper-transmitted, monocot-infecting, monopartite geminiviruses&lt;sup&gt;d&lt;/sup&gt;</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chloris striate mosaic virus&lt;sup&gt;1&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R1</td>
<td>Not infectious</td>
<td>Reduction in ssDNA</td>
<td>Coat protein; Also, needed for spread, insect transmission</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Symptom determinant, may affect streak width</td>
<td></td>
</tr>
<tr>
<td>Digitaria streak virus&lt;sup&gt;2&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R2</td>
<td>Not infectious</td>
<td>Same as wild-type</td>
<td>Required for spread; Symptom determinant, may affect streak width</td>
<td></td>
</tr>
<tr>
<td>Maize streak virus&lt;sup&gt;3&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tobacco yellow dwarf virus&lt;sup&gt;4&lt;/sup&gt;</td>
<td>L1'</td>
<td>Not infectious</td>
<td>No replication</td>
<td></td>
</tr>
<tr>
<td>Wheat dwarf virus&lt;sup&gt;5&lt;/sup&gt;</td>
<td>L1''</td>
<td>Not infectious</td>
<td>No replication</td>
<td></td>
</tr>
<tr>
<td><strong>Subgroup II: Leafhopper-transmitted, dicot-infecting, monopartite geminiviruses</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Beet curly top virus (CFH&lt;sup&gt;6&lt;/sup&gt;, California&lt;sup&gt;7&lt;/sup&gt; and Logan&lt;sup&gt;8&lt;/sup&gt; strains)</td>
<td>R1</td>
<td>Not infectious</td>
<td>Reduction in ssDNA</td>
<td>Same as AR1 (?)</td>
</tr>
<tr>
<td></td>
<td>R2</td>
<td>Infectious but symptomless, Reduced ssDNA</td>
<td>Altered ratio of viral DNA forms</td>
<td>Symptom determinant; Regulates ss-/dsDNA ratio, (controls RF)RF to RF)SS switch?</td>
</tr>
<tr>
<td></td>
<td>R3</td>
<td>Weakly infectious (reduced DNA, no symptoms)</td>
<td>Same as wild-type</td>
<td>Needed for virus transport</td>
</tr>
<tr>
<td></td>
<td>L1</td>
<td>Not infectious</td>
<td>Lack of replication</td>
<td>Same as AL1 (?)</td>
</tr>
<tr>
<td></td>
<td>L2</td>
<td>Same as L3</td>
<td>Same as wild-type</td>
<td>No clear function can be assigned to L2; Does not trans-activate R1; Phenotype in plants may be a result of effect on L3</td>
</tr>
<tr>
<td></td>
<td>L3</td>
<td>Attenuated symptoms, &quot;recovery&quot; phenotype</td>
<td>Reduced levels of ss- and dsDNA</td>
<td>Same as AL3 (?)</td>
</tr>
<tr>
<td></td>
<td>L4</td>
<td>Symptom variability</td>
<td>ND</td>
<td>Symptom determinant</td>
</tr>
</tbody>
</table>
"TABLE 1 (continued)"

| Subgroup III: Whitefly-transmitted, dicot-infecting, bipartite geminiviruses |
|---------------------------------|-----------------|-----------------|-------------------------------------------------|
| African cassava mosaic virus*    | AR1             | Attenuated symptoms | Reduction in ssDNA                                 |
| Abutilon mosaic virus**         | AL1             | Not infectious      | No replication                                    |
| Bean golden mosaic virus**      | AL1             | Not infectious      | No replication                                    |
| Potato yellow mosaic virus**    | AL2             | Absence of viral DNA, | Reduction in ssDNA,                              |
| Squash leaf curl virus**        | AL3             | symptoms            | coat protein levels                               |
| Tomato leaf curl virus**        | AL4             | Wild-type           | Wild-type                                         |
| Tomato yellow leaf curl virus** | BR1             | Not infectious      | Same as wild-type                                 |
| (Israeli* and Thailand** isolate) | BL1             | Not infectious      | Same as wild-type                                 |

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The results of numerous studies, many of which are referenced in the text, are summarized in this Table.

Abbreviations and references for the nucleotide sequences are: 1 CSV (Andersen et al., 1988), 2 DSV (Donson et al., 1987), 3 MSV (Mullineaux et al., 1984; Lazarowicz, 1988), 4 TobYDV (Morris et al., 1992), 5 WDV (MacDowell et al., 1985), 6 BCTV-California (Stanley et al., 1986), 7 BCTV-CFH (personal communication), BCTV-Logan (Chapter II), 8 ACMV (Stanley et al., 1983), 9 AbMV (Frischmuth et al., 1990), 10 BGMV (Howarth et al., 1985), 11 PYMV (Coutts et al., 1991), 12 SqLCV (Lazarowicz et al., 1991), 13 TGMV (Hamilton et al., 1984), 14 TLCV (Dry et al., sequence not published), 15 TYLCV (Navot et al., 1991), 17 (Rochester et al., 1990)  

Gene nomenclature may vary for viruses within a subgroup. For subgroup I, II and III, the MSV (South African isolate), BCTV, and TGMV nomenclatures are used.

TobYDV is the only dicot-infecting member of this subgroup. It is placed in this subgroup because of its genome organization; it lacks the AL2, AL3, AL4 genes and the gene function encoded by AL1 is split into amino- and carboxy-terminal ORFs similar to the other members of subgroup I.

TLCV and the Israeli isolate of TYLCV lack a B component. The Thailand isolate of this virus produces severe symptoms even in the absence of its B components. However, infections in the presence of the B component are more pronounced.
require the coat protein. Instead, the BR1 protein, which shows sequence homology with the coat protein (AR1; Kikuno et al., 1984), and the unrelated BL1 protein are required. It is possible that the coat protein or a capsid-like protein (BR1) is needed in order to coat or bind ssDNA and, therefore geminivirus transport may be similar to the movement proteins of TMV or CaMV in this respect. However, aside from the recent finding that the BL1 protein of bipartite geminiviruses is present in cell wall fractions from infected plants (Pascal et al., 1993; von Arnim and Stanley, 1993), and the limited sequence similarity of geminivirus and RNA virus movement proteins (Koonin et al., 1991), it is not clear whether geminiviruses adhere to the model for plant virus movement proposed by Citovsky and Zambryski (1991). Experiments addressing the mechanism by which geminiviruses are transported within the plant body have yet to be conducted. (Elmer et al., 1988a; Hayes et al., 1989; Hanley-Bowdoin et al., 1990).

The involvement of a protein which may bind ssDNA appears to be the only factor common in the transport of the three subgroups of geminiviruses. The amino acid sequences of the BL1, R2, and R3 proteins are extremely dissimilar. It is probable that different movement mechanisms have been adopted by the three subgroups of geminiviruses, accounting for the absence of sequence similarities. Certainly, differences pertaining to host type, such as monocot (subgroup I) vs dicot (subgroups II and III), and restriction of the infection to phloem tissue seen mainly with geminiviruses belonging to subgroups I and II, lend support to this idea.

Geminivirus Replication. In general, studies on geminivirus replication have followed two different but complementary directions. These are, (1) the identification of proteins which may be part of a complex needed for replication, and (2) the identification of an origin of replication, which serves to initiate + strand synthesis.
AL1 is the only conserved ORF found in all geminivirus genomes irrespective of subgroup affiliation. In subgroup I geminiviruses a protein homologous to AL1 is formed from a spliced mRNA which combines sequences from the overlapping L1' and L1" ORFs (Schalk et al., 1989; Ugaki et al., 1991). Thus, the amino-terminal segment of the protein is derived from the L1' ORF, whereas the carboxy-terminal segment is derived from the L1" ORF which is located in a different reading frame. The first observation that AL1 protein alone is required for replication was demonstrated by Elmer et al. (1988). Later studies by Hayes et al. (1989) and Hanley-Bowdoin et al. (1990) using transgenic plants showed that AL1 was both necessary and sufficient for replication. Interestingly, sequence comparisons suggest that the AL1 protein is related to the rolling circle initiator proteins found on the genomes of pC194-like plasmids, and that it may be an ATP-dependent helicase (Gorbalenya et al., 1990). These data are consistent with the hypothesis that AL1 initiates ssDNA synthesis by introducing a specific nick at the viral origin of replication, as occurs in the ssDNA phages. Mutagenesis studies also indicate that the AL3 protein serves to enhance replication levels (Sunter et al., 1990; Etessami et al., 1991), but the means by which it influences replication has yet to be discovered. In addition, geminivirus coat protein (AR1) mutants (Briddon et al., 1989; Sunter et al., 1990; Etessami et al., 1991; Boulton et al., 1993; Hormuzdi and Bisaro, 1993) and AL2 mutants (Sunter et al., 1990) accumulate only reduced levels of ssDNA, possibly because they are required directly or indirectly for protection of viral DNA from nucleases. Mutations in the AL2 gene were found to abolish infectivity in plants (Bisaro et al., 1990; Etessami et al., 1991; Morris et al., 1991, Elmer, 1988a), although the gene is not necessary for replication since AL2 mutants in protoplasts, like coat protein mutants, replicate dsDNA to wild-type levels but accumulate reduced levels of ssDNA (Sunter et al., 1990). The discovery that TGMV AL2
protein transactivates AR1 gene expression explained the similar phenotype seen in protoplasts for AL2 and AR1 mutations (Sunter and Bisaro, 1991). The inability of AL2 mutants to infect plants may be attributed to the requirement of the AL2 protein for transactivation of BR1, a movement protein (Sunter and Bisaro, 1992). The ability of the AL2 gene product to transactivate the AR1 and BR1 genes has since been demonstrated for ACMV (Haley et al., 1992).

Within the intergenic region of all geminiviruses sequenced to date is a ~30 nucleotide region bordered by inverted repeats. This conserved region is capable of forming a stable hairpin structure (Sunter et al., 1985) whose loop contains an invariant sequence (TAATATTAC) that is similar to the sequence surrounding the replication initiator protein cleavage sites in bacteriophage φX174 (Goetz et al., 1988) and plasmid pC194 (Michel et al., 1986). Mutational analysis has demonstrated that the sequence encompassing the inverted repeat is required for geminivirus replication (Revington et al., 1989; Lazarowitz et al., 1992), and an origin of replication has been localized to a larger region which includes sequences 5’ of the inverted repeat (Lazarowitz et al., 1992). Analysis of viral DNA replicated from beet curly top recombinants composed of different strains has suggested that the site of single-strand cleavage and subsequent initiation of rolling circle replication lies within the inverted repeat (Stenger et al., 1991). Recent demonstrations that the AL1 gene product binds specifically to dsDNA sequences near the hairpin (Fontes et al., 1992), and to ssDNA with greater affinity (Thommes et al., 1993), support the notion that it may be the protein responsible for cleavage.
OBJECTIVES OF RESEARCH

The analysis of virus mutants in single-cell and whole-plant systems is a powerful genetic approach to categorize gene products into those required either for movement or for replication. This approach has been used very effectively in understanding geminivirus gene function (see Table 1) and preliminary studies of this nature have often pointed research projects in successful directions.

The experiments described in the following chapters utilize the genetic approach described above to assess the contribution of TGMV BL1 and BR1 genes to movement, and to investigate the functions of the R1, R2, R3, L2 and L3 genes of BCTV. A careful and systematic study of these genes, such as has been described in the subsequent chapters, was expected to indicate whether these genes participate in replication, spread, and/or symptom development. Studies of this nature will lay the groundwork for future investigations into the mechanisms by which viral gene products interact with host components and carry out their functions. Ultimately, a knowledge of these interactions is necessary if we are to understand in molecular detail the interactions between host and pathogen that result in disease.
CHAPTER I

STUDIES ON MOVEMENT OF TOMATO GOLDEN MOSAIC VIRUS:

REQUIREMENT OF THE B COMPONENT GENES FOR SYSTEMIC AND

CELL-TO-CELL SPREAD

INTRODUCTION

Geminiviruses are divided into three subgroups based upon their genome structure and means of transmission. Differences between the subgroups are reflected in the presence and organization of ORFs on the genome and are evident in the different strategies adopted by subgroup members for replication of the genome, regulation of viral gene expression and spread of the virus in the infected plant. Examples of such differences include transactivation of coat protein gene expression by AL2 protein in members of subgroup III (Sunter and Bisaro, 1991; Haley et al., 1992; Sunter and Bisaro, 1992) and by L1 protein for members of subgroup I (Hofer et al., 1992), and the involvement of a virion sense gene in the regulation of ss- and dsDNA levels in BCTV (Hormuzdi and Bisaro, 1993). Geminiviruses have also evolved differently with respect to the genes involved in transport (refer to Table 1 and Introduction chapter).

It was demonstrated that transgenic plants carrying chromosomal inserts of the TGMV A component were symptomless and yet contained free extrachromosomal ss- and dsDNAs specific for the A component (Rogers et al., 1986). These transgenic
plants were also shown to contain virus particles (Sunter et al., 1987). This indicated that the A component encodes all the genetic information required for viral DNA replication and encapsidation. However, both the A and B components are necessary to produce a symptomatic infection in wild-type, non-transgenic plants (Hamilton et al., 1983). Likewise, transgenic plants containing chromosomal copies of both TGMV DNA A and DNA B showed disease symptoms and contained replicating extrachromosomal DNA of both the genome components. It was therefore suggested that the failure of plants inoculated with TGMV A to show disease symptoms was due to the lack of movement functions located on the B genome (Rogers et al., 1986). Mutagenesis experiments have since been conducted on the B components of several bipartite geminiviruses (Brough et al., 1988; Etessami et al., 1988; Sunter et al., 1990; Evans and Jeske, 1993) in order to examine the participation of the BL1 and BR1 ORFs in virus spread and symptom development.

The studies described in this chapter were aimed at understanding the involvement of the B component genome in facilitating TGMV movement. Some of the studies described were part of a larger effort aimed at elucidating the roles of the various reading frames located on the A and B components of TGMV (Bisaro et al., 1990; Sunter et al., 1990). In addition, the ability of the A genome to spread in the absence of the B genome if delivered directly to the phloem tissues of the plant has also been investigated. This experiment was conducted since recent reports (Klinkenberg et al., 1990; Evans and Jeske, 1993) have indicated that the A component of bipartite geminiviruses is capable of producing a weak, symptomless infection under some conditions. The implications of these results for transport of bipartite geminiviruses is discussed.
MATERIALS AND METHODS

DNA techniques. All restriction endonucleases and other DNA modifying enzymes were obtained from Promega (Madison, WI) and used as specified by the supplier. Other techniques were performed essentially as described by Ausubel et al. (1987). All TGMV nucleotide coordinates used refer to the nucleotide sequence of Hamilton et al. (1984) as modified by MacDowell et al. (1986).

Preparation of TGMV mutants. For a schematic description of the genomes used to transfect protoplasts refer to Figure 5. A mutation in the BR1 ORF was constructed by restriction of DNA B with EcoRI at nucleotide 651, followed by end-fill using the Klenow fragment of DNA Polymerase I and religation in the presence of T4 DNA ligase. The mutation was confirmed by demonstrating the absence of an EcoRI site and the presence of a new XmnI site. The mutation is expected to produce a truncated BR1 protein consisting of the N-terminal 62 amino acids fused to 6 amino acids from a different reading frame. A mutation within the BL1 ORF was constructed by restriction of DNA B with NcoI at nucleotide 1857, followed by end-fill and religation. The mutation was confirmed by the absence of an NcoI site and presence of a new NsiI site. The mutation is expected to produce a truncated BL1 protein consisting of the N-terminal 114 amino acids fused to 25 amino acids from a different reading frame.

Unit-length wild-type or mutant TGMV DNA B genomes were cloned into pUC118-based vectors (Vieira et al., 1987) containing the 1255 bp EcoRI-BglII fragment of DNA B (pTGB38). The wild-type and mutant TGMV B constructs were inserted at the Clal site of pTGB38 to obtain tandemly repeated 1.5 constructs containing two common regions. These constructs are shown in Figure 5. The B
FIGURE 5. Diagrams of TGMV A and B genome components and mutants. (A) The two double-stranded replicative form DNAs of TGMV are drawn from the sequence of Hamilton et al. (1984) and MacDowell et al. (1986). The solid arrows define the positions of open reading frames and the hatched box indicates the region common to both components. Positions of restriction sites are indicated outside the circles. (B) The two circles illustrate pUC118 containing either the 1128 bp EcoRI-ScaI fragment of DNA A (pTGA20) or the 1255 bp BglII-EcoRI fragment of DNA B (pTGB38). The pUC118 sequences are indicated by the thin lines and the TGMV sequences by thick lines. The shaded box on the circles represents the 230 bp common region. The linear maps illustrate unit-length TGMV DNAs inserted into either the EcoRI or Clal sites of pTGA20 or pTGA38, respectively. The hatched box on each line represents the common region and the boxes above each line represent individual ORFs. The shading within ORF boxes illustrates the uninterrupted reading frame. The triangles indicate the positions of insertions.
“FIGURE 5 (continued)”

B

![Diagram of molecular clones](image-url)
component mutants have also been inserted into Ti plasmid pMON505-based vectors containing the 940 bp $\text{ClaI}-\text{BglII}$ fragment of DNA B (Elmer et al., 1988b). The relevant pMON505 derivatives are: wild-type A, pMON337; wild-type B, pMON393; BR1-, pTGB12; BL1-, pTGB37.

**Inoculation of plants.** Healthy *Nicotiana benthamiana* plants at the six-leaf to eight-leaf stage were inoculated at the shoot tip with Agrobacterium cultures (agroinoculation; Grimsley et al., 1986; Grimsley and Bisaro, 1987). Shoot tips were excised with a scalpel and the inoculum was then applied to the cut stem. The pin-pricking method of inoculation was essentially the same except that the cut stem was pricked multiple times with an insect pin after the application of the inoculum.

**Protoplast transfection.** Protoplasts were prepared from *N. tabacum* cv. Wisconsin suspension culture cells (a gift of Dr. D. P. S. Verma) and transfected essentially as described by Potrykus and Shillito (1986) and Negrutiu et al. (1987), with minor modifications (Brough et al., 1992; Inamdar et al., 1992).

**DNA isolation and analysis.** DNA from protoplasts and plants was isolated according to the procedure of Mettler (1987), and quantified by spectrophotometry. Samples containing DNA were electrophoresed through 1% agarose gel, transferred to Nytran membrane (Schleicher and Schuell, Keene, NH) and analyzed by hybridization with $^{32}\text{P}$-labeled riboprobes prepared from TGMV clones in pGEM vectors (Promega, Madison, WI) as described previously (Sunter et al., 1989). Quantification of DNA on Southern blots was carried out using a radioanalytic imaging system (AMBIS, San Diego, CA).
Western blots analysis. Protein extracts were obtained from protoplasts transfected with TGMV constructs. Total crude proteins were precipitated at -20 C from the phenol/chloroform phase obtained during the isolation of DNA by the addition of 5 volumes of 95% methanol/0.1M ammonium acetate. Following recovery the protein was washed with acetone, dried and resuspended in 1X Laemmli buffer. Protein concentrations were determined by the method of Bradford (1976).

Protein samples were electrophoresed through 12% polyacrylamide resolving gels under denaturing conditions as described by Laemmli (1970). The protein was then transferred to nitrocellulose membrane using the electrotransfer apparatus and protocol of Idea Scientific (Corvallis, OR). Following transfer the membrane was blocked and then incubated for 16-20 hr with antibody prepared against TGMV virions (1:10,000 dilution; Sunter et al., 1990). Following incubation of the membrane with the secondary antibody (goat anti-rabbit conjugated to alkaline phosphatase) the blot was developed using nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) as substrates. Development was stopped by the addition of water.

RESULTS

TGMV B ORF mutants and their assay in protoplasts. A diagram of the TGMV genome which shows the positions of the two B component ORFs is presented in Figure 5. Mutations were constructed within each of these ORFs, and viral genomes carrying ORF mutations were introduced into pUC118 vectors containing approximately one-half of the TGMV B component. The constructions used to transfect protoplasts therefore contain a partially duplicated viral genome, but only one copy of the mutated sequence.
<table>
<thead>
<tr>
<th>DNA Form</th>
<th>DNA A</th>
<th>DNA B</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>pTGA26 + pTGB43</td>
<td>pTGA26 + pTGB42</td>
</tr>
<tr>
<td># Expts</td>
<td>Range</td>
<td>Avg.</td>
</tr>
<tr>
<td>dsDNA</td>
<td>3</td>
<td>1.25 - 2.00</td>
</tr>
<tr>
<td>ssDNA</td>
<td>3</td>
<td>1.40 - 2.25</td>
</tr>
<tr>
<td>Total DNA</td>
<td>3</td>
<td>1.35 - 2.00</td>
</tr>
</tbody>
</table>

The values given represent the amount of DNA A or DNA B in protoplasts transfected with DNA B mutants relative to wild-type DNA levels. Wild-type DNA levels were arbitrarily assigned a value of 1. Values used to calculate total DNA levels were adjusted to account for strandedness.
FIGURE 6. Southern blot analysis of DNA B mutant DNAs isolated from transfected protoplasts. DNA isolated from transfected protoplasts was hybridized to $^{32}$P-labeled DNA A- or B-specific probes. Each lane contains 1 µg of DNA from protoplasts transfected with wild-type DNA A and the following DNA B constructs: wild-type DNA B (lanes 1 and 5), BR1- (lanes 2 and 6) or BL1- (lanes 3 and 7). Also shown is DNA from plants infected with TGMV (lanes 4 and 8). Panels hybridized with DNA A- or DNA B-specific probes are indicated.
Mutant constructions were transfected into *N. tabacum* suspension culture-derived protoplasts, and their replication compared with wild-type controls containing 1.5 copies of TGMV A or B DNA in the pUC118 vector (pTGA26 and pTGB40, respectively; Fig. 5). As DNA B cannot replicate independently, protoplasts were co-transfected with pTGA26 and pTGB40 or the DNA B mutants. The extent of replication was determined by Southern blot analysis of protoplast DNA isolated 3-5 days post-transfection.

**Phenotype of DNA B mutants in protoplasts.** Disruption of either reading frame did not affect the replication of either the B genome or the co-transfected A genome. As shown in Figure 6, BR1 (pTGB43; lanes 2 and 6) or BL1 (pTGB42; lanes 3 and 7) mutants replicated as well as the wild-type genome (lanes 1 and 5) as judged by the quantity and relative abundance of ss- and dsDNA forms of the A and B genomes. Quantitative data for a number of experiments are presented in Table 2 and support this contention.

Proteins extracted from protoplasts transfected with the B mutants were analyzed on western blots probed with antiserum raised against purified TGMV virions. As shown in Figure 7, a major polypeptide of approximately 29 kD, which corresponds to native TGMV coat protein (lane 5), was detected in protoplasts transfected with wild-type, BR1- and BL1- genomes (lanes 1, 2 and 3 respectively), indicating that mutations within the B component ORFs do not affect coat protein accumulation.

**The DNA B ORFs are required for infectivity.** B component genomes bearing disruptions in either BL1 (pTGB37) or BR1 (pTGB12) ORFs were inserted into Ti plasmid vectors and analyzed for their ability to infect *N. benthamiana* plants.
FIGURE 7. Western blot analysis of proteins isolated from transfected protoplasts. Total protein extracts from protoplasts were analyzed on western blots incubated with TGMV coat protein antibody. Protein from protoplasts transfected with the following DNA B constructs are shown: wild-type DNA B (lane 1), BR1- (lane 2), and BL1- (lane 3). Also shown is protein from healthy untransfected protoplasts (lane 4) and from purified TGMV virions (lane 5). The position of TGMV coat protein is indicated.
Each of the mutants was agroinoculated along with wild-type TGMV A (pMON337); the plants were inspected for symptoms and the presence of freely replicating TGMV DNA A and B forms by Southern blot analysis. The results of these experiments (data not shown) indicated that plants inoculated with the mutant genomes showed neither symptoms nor the presence of freely replicating viral DNA. On the other hand, control plants inoculated with wild-type DNA A and DNA B components showed typical TGMV disease symptoms and contained extrachromosomal forms of TGMV DNA (for example see Fig. 8, lane 1).

**Spread of the A genome in the absence of the B component.** Recent studies with African cassava mosaic virus (ACMV; Klinkenberg and Stanley, 1990) and abutilon mosaic virus (AbMV; Evans and Jeske, 1993) showed that the A component of these viruses can spread to a limited extent even in the absence of the B component when inoculated by the pin-pricking method. This is the predominant method of inoculation used for monopartite geminiviruses and presumably delivers the inoculum directly to the phloem. In order to determine whether TGMV DNA A also can infect plants in the absence of DNA B, *N. benthamiana* plants were pin-prick inoculated with pMON337. The plants so inoculated were symptomless, although Southern blot analysis of DNA extracted from fourteen plants indicated that nine contained detectable levels of TGMV A DNA (Fig. 8). The levels of viral ss- and dsDNA observed in pMON337 inoculated plants were much lower than the levels seen in plants inoculated with both components of the TGMV genome.
FIGURE 8. Southern blot analysis of DNA isolated from plants infected with TGMV A DNA. Total DNA isolated from wild-type TGMV DNA A and DNA B infected (lane 1), uninfected healthy (lane 2) and TGMV DNA A infected (lanes 3-16) *N. benthamiana* plants was subjected to Southern blot analysis as described before. Each lane contains 10 µg of DNA. Also shown is TGMV DNA marker (lane 18) indicating the positions of open circular (OC), supercoiled (SC) and single-stranded (SS) DNA forms of the freely replicating, unit-length TGMV.
DISCUSSION

The studies described in this chapter assess the requirement of the BL1 and BR1 ORFs for replication and spread of TGMV. These studies followed experiments which indicated that both genome components were required for a symptomatic and systemic infection (Hamilton et al., 1983; Stanley, 1983). Utilizing plants transgenic for the A genome component of TGMV, Rogers et al. (1986) were able to show that the A component encodes all the functions necessary for replication and proposed that the B component encodes functions essential for symptom production and virus spread.

Independent Spread of TGMV DNA A. The data presented in Figure 8 indicate that DNA A of TGMV is capable of movement independent of DNA B. Thus, TGMV is similar to ACMV (Klinkenberg and Stanley, 1990) and AbMV (Evans and Jeske, 1993) in this regard. However, in each of these studies the amount of viral DNA was observed to be much lower than in a normal wild-type infection, and the infected plants were asymptomatic. Thus, although the A genome may possess a means of limited spread, the B genome is required for symptom production and efficient movement, resulting in amplification of viral DNA levels. The AL1 and AL2 gene products, for which a direct role in movement cannot be ruled out (see discussion below) may be involved in this form of movement. It is also possible that the coat protein (AR1), which shows sequence homology to the BR1 movement protein (Kikuno et al., 1984), may play a role.

Geminiviruses are divided into three subgroups based on the nature of their transmission and genome structure. The observation that the A component of the bipartite geminiviruses is capable of spread suggests that the distinction between a
one or two molecule genome may not be very rigid. This is also suggested by the recent discovery that different strains of the whitefly-transmitted tomato yellow leaf curl virus (TYLCV) have different requirements with respect to the B component. A strain of TYLCV has been identified which lacks a B component (Navot et al., 1991) and yet is capable of producing a symptomatic infection. Furthermore, it has been shown that DNA A of the strain which does possess a B component is capable of producing a symptomatic infection in the absence of DNA B (Rochester et al., 1990). Thus it is possible that the evolution of the monopartite geminiviruses from the bipartite ones has occurred through the acquisition of genes which enhance the spread and symptom production of the A component. It is also possible that geminivirus evolution has occurred in the reverse direction; that is, by the loss of efficient A component spread following the acquisition of a second genome bearing movement functions.

Requirement and roles of the BR1 and BL1 genes in movement. A protoplast-based system, which allows the replication of plant viral nucleic acids to be studied under more or less synchronous conditions, independent of systemic movement requirements (Motoyoshi, 1985), was used to examine the replication efficiency of mutants bearing disruptions in the BR1 and BL1 ORFs. The mutants were able to replicate well and the lesions did not hinder the ability of the co-transfected A genome to replicate. Furthermore, the data obtained from western blots indicates that these disruptions also did not impair synthesis of coat protein, suggesting that the B component ORFs are not needed for the expression of the coat protein gene or for the assembly of virions. This confirms earlier studies which showed the presence of virus particles in transgenic petunia plants containing tandem direct repeats of the A genome component of TGMV (Sunter et al., 1987).
Although replication competent, the DNA B mutants were incapable of generating an infection when inoculated onto plants along with wild-type DNA A. Therefore, the disruption of BR1 and BL1 appears to abolish efficient cell-to-cell spread and systemic infection of tissues but does not affect the ability of the virus to replicate in single cells. The DNA B gene products may then be formally defined as movement proteins. Earlier mutagenesis studies had also implicated the DNA B gene products in movement of the virus (Brough et al., 1988). However, no one before had looked at possible replication effects of the DNA B gene products. The studies presented in this chapter demonstrate that the lack of infectivity observed with B component ORF mutants is not due to an effect on replication. These and other mutagenesis studies conducted on ACMV (Etessami et al., 1988; von Arnim and Stanley, 1993) and AbMV (Evans and Jeske, 1993) implicate the two reading frames on the B genome component of the bipartite geminiviruses in systemic spread.

Recently it has been suggested that BR1 protein is responsible for cell-to-cell spread, whereas BL1 protein, possibly together with BR1, may be responsible for long distance vascular spread (von Arnim and Stanley, 1993). These conclusions were based on the reduced accumulation of BR1 mutants in agroinoculated leaf discs compared to wild-type virus and BL1 mutants. However, it is possible that the reduction in viral DNA levels seen with the BR1 mutant was due to an inhibitory influence of the BL1 protein, and not on a defect in cell-to-cell spread as suggested by the authors. Two instances of such an inhibitory effect have been noted. Amino-terminal sequences of TGMV BL1 protein have previously been shown to have a dominant negative effect on the spread of coinoculated ACMV (von Arnim and Stanley, 1992). Also, a BR1 AbMV mutant was observed to have a more pronounced negative effect on the spread of the coinoculated A component than a BL1 mutant (Evans and Jeske, 1993). It is possible that in the latter study, the intact BL1 protein
expressed by the BR1 mutant had a negative effect on the putative movement function encoded by the A genome. Clearly any conclusions concerning the involvement of the BL1 and BR1 proteins in localized and long distance spread must be regarded as preliminary.

Mutagenesis has revealed that mutations in ORFs corresponding to the AL1, AL2, BR1 and BL1 ORFs of TGMV abolish infectivity (for review see Stanley, 1991). The AL1 ORF is essential for replication, but the AL2, BR1 and BL1 mutants replicate in single-cell systems and yet are required for infectivity. Therefore, the products of these genes are either directly or indirectly implicated in movement. It has been demonstrated that the AL2 gene product is required for the expression of both the coat protein (AR1) and BR1 genes (Sunter and Bisaro, 1991; Sunter and Bisaro, 1992). Thus, it would appear that the AL2 protein is required for movement because it is needed for the transactivation of BR1 expression, although a direct role in viral transport has not been ruled out (Sunter et al., 1992). The movement genes located on the B component, on the other hand, may be directly involved in the translocation of the genome across the plasmodesmata.

Recently it was shown that the ACMV BL1 protein is localized in the cell wall fraction of infected plant cells (Pascal et al., 1993; von Arnim and Stanley, 1993). This suggests that the BL1 protein, like the movement proteins of TMV and CaMV, is associated with plasmodesmata. A functional homology between these movement proteins has also been implied by a computer analysis (Koonin et al., 1991). Furthermore, cytoplasmic tubular structures that are continuous with the plasmodesmata and which contain virus particles have recently been reported for a geminivirus infection (Kim and Lee, 1992) Such structures are common in infections of comoviruses and caulimoviruses and are suggestive of cell-to-cell movement of virus particles. Immunofluorescence staining of infected cells has revealed that the
CaMV movement protein is a component of the tubular structures (Perbal et al., 1993). It remains to be seen which, if any, of the B component proteins are structural components of the cytopathic tubular structures observed by Kim and Lee (1992).

In conclusion, the evidence suggests that the bipartite geminiviruses and RNA viruses such as TMV and CaMV may share a similar stage(s) in their transport processes, and that the geminivirus B component proteins play a crucial role in facilitating spread of the virus in the infected plant.
CHAPTER II

NUCLEOTIDE SEQUENCE AND ANALYSIS OF THE LOGAN STRAIN
OF BEET CURLY TOP VIRUS

INTRODUCTION

Beet curly top virus (BCTV) is a pathogen of significant economic importance. It is extremely widespread in its distribution and can infect more than 300 species from 44 plant families (Bennett, 1971). Its host range is restricted to dicotyledonous plants although the leafhopper vector responsible for its transmission, 
Circulifer tenellus, feeds readily on a number of monocots. Typical strains of BCTV have a wide host range and induce severe disease of sugarbeet, Beta vulgaris L. However, many phenotypic variants of BCTV have been described (Bennett, 1971; Stenger et al., 1990) which differ with respect to host range and severity of symptoms. An examination of the similarities and differences in nucleotide sequences between the different strains of BCTV would facilitate the identification of sequences controlling the expression of symptoms and host range.

The Logan strain of BCTV has been used in the studies described in chapters II, III, and IV. This work has involved the manipulation of genomic sequences for mutational and cloning purposes. The manipulations required for these studies were made possible after a determination of the nucleotide sequence of the Logan strain, which is reported in this chapter. Also discussed are various features of the
nucleotide sequence, the similarity of the predicted gene products with those located on other geminivirus genomes, and the pathology of the virus.

MATERIALS AND METHODS

DNA techniques. All restriction endonucleases and other DNA modifying enzymes were used as specified by the supplier. Other techniques were performed essentially as described by Ausubel et al. (1987), and Maniatis et al. (1982).

Source of the BCTV-Logan clone. The construction of plasmid pLogan, which contains BCTV-Logan DNA cloned as a SalI fragment in pUC8, has been reported previously (Stenger et al., 1992). This plasmid was kindly provided by Dr. D.C. Stenger and was used as the source of BCTV genomic DNA for the manipulations described in chapters II, III, and IV.

Construction of clones for infectivity studies. A restriction map of BCTV-Logan, indicating the positions of restriction enzyme sites used for cloning and mutagenesis, is shown in Figure 9.

Plasmids pCT1 and pCT3 were constructed by inserting the 1921 bp and 1117 bp SalI-DraI BCTV DNA fragments, respectively, into SalI and SmaI-digested pIC20H (Marsh et al., 1984). The full-length (~3 kbp) BCTV genome was then inserted into the SalI site of these plasmids. The resulting plasmids, which contain approximately 1.5 tandemly repeated copies of BCTV DNA, were designated pCT2 and pCT4. pCT2, which is the larger of the two, contains two copies of the intergenic region (IR), whereas pCT4 contains only a single copy. In order to investigate the importance of an ApaI containing triple repeat, plasmid pCT15, which contains only a
FIGURE 9. Map of the BCTV-Logan genome. The genome of BCTV is represented as the dsDNA replicative form. Shown are the seven ORFs located on the genome (indicated by arrows). The direction of the arrow indicates the strandedness of the ORFs; clockwise oriented arrows represent ORFs on the virion strand and anticlockwise arrows indicate ORFs located on the complementary strand. The positions of selected restriction enzyme cleavage sites are shown along with their nucleotide coordinates (in parenthesis). Also indicated is the intergenic region (IR) which contains the conserved hairpin sequence whose loop contains the SspI site (nucleotide coordinate 97).
single copy of this sequence, was constructed. This was accomplished by restricting pLogan with ApaI, which releases a 1963 bp BCTV fragment, two copies of the repeat and a larger DNA fragment consisting of vector and BCTV sequences. The 1963 bp ApaI fragment was then ligated back to the fragment containing pIC20H DNA using T4 DNA Ligase. Thus, pLogan lacking two copies of the 36 nucleotide ApaI repeat was created. The SalI BCTV DNA fragment from pCT15 was inserted into pCT3 to generate pCT17.

For each of the constructs in pIC20H, a HindIII to ClaI fragment containing the tandem BCTV repeats was inserted into the Ti plasmid vector pMON521 (a derivative of pMON505; Rogers et al., 1987). The relevant pMON521 derivatives are: pCT6, pCT8 and pCT20, which are derived from pCT4, pCT2, and pCT17, respectively. These constructs were used to introduce the viral genome into plants by the method of agroinoculation (Grimsley et al., 1986; Grimsley and Bisaro, 1987).

BCTV-Logan DNA, obtained as a SalI fragment from pLogan, was cloned in opposite orientations in pBluescript SK+ (Stratagene, La Jolla, CA). The resultant plasmids, called pCT9 and pCT10, were used in the Riboprobe System (Promega, Madison, WI) to obtain strand-specific probes. In this method radiolabeled ribonucleotides are incorporated into a single-stranded nucleic acid molecule by the activities of SP6, T7, or T4 RNA Polymerase. Thus, single-stranded radiolabeled RNAs are synthesized which may be used in hybridization procedures to differentiate between viral and complementary sense strands. Probes having the same sense as virion DNA, and hence capable of hybridizing to complementary sense DNA, were obtained using pCT9, whereas probes for virion sense DNA were obtained using pCT10.
Inoculation of plants. Healthy *Nicotiana benthamiana* plants at the six-leaf to eight-leaf stage were inoculated at the shoot tip with Agrobacterium cultures (agroinoculation; Grimsley et al., 1986; Grimsley and Bisaro, 1987). Shoot tips were excised with a scalpel and the inoculum was applied to the cut stem. The cut stem was then pricked multiple times with an insect pin. The inoculation procedure employed for sugarbeet plants was essentially the same except that the inoculum was applied to the intact apical region of the plant.

Protoplast transfection. Protoplasts were prepared from *N. tabacum* cv. Wisconsin suspension culture cells (a gift of Dr. D. P. S. Verma) and transfected essentially as described by Potrykus and Shillito (1986) and Negrutiu et al. (1987), with minor modifications (Brough et al., 1992; Inamdar et al., 1992).

DNA isolation and analysis. DNA from protoplasts and plants was isolated according to the procedure of Mettler (1987), and quantified by spectrophotometry. Samples containing DNA were electrophoresed through 1% agarose gel, transferred to Nytran membrane (Schleicher and Schuell, Keene, NH) and analyzed by hybridization with 32P-labeled riboprobes prepared from BCTV clones in pBluescript SK+ vectors (Stratagene, La Jolla, CA).

Nucleotide sequence of BCTV. The DNA sequencing procedures specified in the DNA Sequencing Guide (United States Biochemical Corporation, Cleveland, OH) for use with the Sequenase DNA sequencing kits were followed. This protocol, which involves the use of a modified T7 DNA Polymerase enzyme, is based upon the chain-termination sequencing method of Sanger (1977). A number of clones were sequenced (summarized in Fig. 10). A large number of the sequenced clones were
obtained by subcloning fragments between restriction sites (Fig. 10). In addition, the Erase-a-Base kit (Promega, Madison, WI), which employs a procedure based upon the resistance of DNA ends with 4 bp or larger 3' overhangs to exonuclease III activity, was used to generate nested deletions. Thus, a combination of restriction fragment subcloning and Exonuclease III mediated deletion was used to obtain clones for sequencing BCTV genomic DNA.

**FIGURE 10.** Map of subclones used to determine the sequence of BCTV-Logan. The 3038 bp Logan genome is represented by a thick line. The arrows represent individual subclones. Subclones whose sequence was used to determine the virion strand sequence point in the rightward direction, whereas the leftward arrows indicate subclones which were used to read the sequence of the complementary strand. The positions of restriction enzyme sites are indicated below the BCTV genome.

**Computer analysis of sequence data.** Analysis of the BCTV nucleotide sequence was conducted using the program SeqApp1.9a. ClustalV, which is an external sequence analysis program linked to SeqApp1.9a, was used to align BCTV proteins and to obtain similarity scores. These scores are based on the Dayhoff PAM 250 protein weight matrix and are generated by the method of Wilbur and Lipman (1983).
RESULTS AND DISCUSSION

Infectivity of BCTV-Logan. Typical symptoms indicative of a curly top infection were produced following agroinoculation of sugarbeet and *N. benthamiana* plants with BCTV genomes containing 1.5 tandemly repeated copies of BCTV-Logan genome (pCT6 and pCT8). In *N. benthamiana*, these symptoms include rolling of the leaf edges, clustering of closely packed, dwarfed leaves at the top of the stem, and a distorted and swollen stem. A photograph of an uninoculated plant and a BCTV inoculated plant are shown in Figure 11.

DNA was isolated from infected plants by the method of Mettler (1987) and subjected to Southern blot analysis for the purpose of identifying and determining the nature of BCTV-specific DNAs. Probes of either virion or complementary sense prepared by the Riboprobe system (Promega, Madison, WI) were used to detect BCTV DNAs. Viral dsDNAs are expected to hybridize to both probes, whereas ssDNAs of virion sense should be detected only by a complementary sense probe. As shown in Figure 12, both ss- and ds BCTV-specific DNA of the expected size and conformation were found in infected plants. Thus, monomeric forms of the viral genome were released from the 1.5 copies present in the inoculum DNA. In addition, a number of subgenomic forms were produced in BCTV infections, and these were more varied and abundant in *N. benthamiana* plants than in infected sugarbeet plants, an observation which has also been noted earlier (Frischmuth et al., 1992; Stenger et al., 1992).

The BCTV DNA forms were further characterized by enzyme treatment in conjunction with strand-specific hybridization, as before (Figure 13). The DNA-specific nature of the DNAs was confirmed by their resistance to RNase A and sensitivity to DNase I. Treatment with the single-strand specific S1 nuclease verified
FIGURE 11. Symptoms of wild-type BCTV Logan on N. benthamiana. Photographs of BCTV inoculated (WT) and uninoculated (MOCK) N. benthamiana are shown.
FIGURE 12. Presence of BCTV DNA in inoculated tissue. Five μg of DNA isolated from *N. benthamiana* (NB) or sugarbeet (SB) plants infected with BCTV (pCT6 or pCT8 inoculum) was run on an agarose gel and subjected to Southern blot analysis. DNA in panel A was hybridized to complementary sense probe, whereas DNA in panel B was hybridized to virion sense probe. The positions of open circular (OC), linear (LIN), supercoiled (SC), single-stranded (SS), and subgenomic (SG) forms of BCTV are indicated.
the identity of genomic and subgenomic ssDNAs. Since these species were detected only in blots hybridized with a complementary sense probe, these DNA molecules are all virion (+) sense. The restriction enzyme ScaI was used to linearize dsDNAs in the sample. This enzyme was chosen because there is a unique ScaI site in the BCTV-Logan genome which is located close to the putative plus strand origin of replication, and because previous studies have indicated that this region is maintained in subgenomic DNAs (Frischmuth and Stanley, 1992; Stenger et al., 1992). The positions of subgenomic ss- and dsDNAs identified in this manner are indicated by arrows in Figure 13. Based upon the mobilities of the linear fragments, it is estimated that the subgenomic DNAs present in the infected plant shown in Figure 13 are approximately 1.8, 1.7, 1.5, 1.3 and 0.4 kb in size. Subgenomic DNAs of a similar size have been observed in previous studies (Frischmuth and Stanley, 1992; Stenger et al., 1992). Subgenomic DNAs produced in ACMV and TGMV infected plants have been shown to reduce viral replication and infectivity (Stanley et al., 1985; MacDowell et al., 1986; Frischmuth et al., 1991) and, in the case of ACMV, to attenuate disease symptoms (Stanley et al., 1990). Thus, these subgenomic DNAs are considered to be defective interfering molecules.

Individual subgenomic DNA populations produced in different plants are known to be variable, with some forms appearing in some plants but not in others. The nucleotide sequence of seven subgenomic DNAs found in BCTV infected plants have been determined (Frischmuth and Stanley, 1992; Stenger et al., 1992). A 355 nucleotide region spanning the 5' end of L1 and most of the intergenic region (IR) was found to be common to all of the sequences (Frischmuth and Stanley, 1992). All of the sequenced subgenomic DNAs contained deletions within the L1 gene which has been demonstrated to be necessary for replication of BCTV (Briddon et al., 1989). Therefore, subgenomic DNAs must also be replicated by complexes
FIGURE 13. Verification of BCTV DNA forms. Five μg of DNA isolated from a single infected *N. benthamiana* plant (pCT6 inoculum, same as in Figure 11) was run on an agarose gel and subjected to Southern blot analysis as described. DNA in panel A was hybridized to virion sense probe, whereas DNA in panel B was hybridized to complementary sense probe. Lanes containing untreated samples, or samples which were treated with *Sca*I, *Sac*I and S1 nuclease, DNase I, and RNase A are indicated. Also indicated are the positions of open circular (OC), linear (LIN), supercoiled (SC), and single-stranded (SS) forms of genomic BCTV DNA. The positions of *Sca*I linearized (panel A) and single-stranded (panel B) forms of subgenomic DNA are indicated by arrows.
responsible for the replication of viral DNA, and it is reasonable to assume that the 355 nucleotide segment contains all of the cisacting signals necessary for viral DNA replication. The 0.4 kb subgenomic DNA (Fig. 13) is approximately 150 nucleotides smaller than the smallest subgenome previously identified and about 450 nucleotides smaller than the smallest subgenome previously sequenced. These observations suggest that this truncated genome consists of sequence elements necessary for replication. It is possible that cisacting signals can be further delimited by the cloning and sequencing of small subgenomic DNAs.

Nucleotide Sequence of BCTV-Logan. The nucleotide sequence of the Logan genome was determined and found to be 3038 bp in length (Fig. 14). In keeping with the convention for numbering geminivirus sequences, nucleotide 1 is designated as the first nucleotide upstream of the ORF L1 start codon.

The sequences of the California (Stanley et al., 1986) and CFH (D.C. Stenger, personal communication) strains of BCTV are also known. A DNA homology matrix analysis of BCTV-Logan with BCTV-CFH and BCTV-California was generated using a window size of 20 nucleotides and a maximum allowable 5 mismatches per window (Fig. 15). This analysis allows a comparison of the three genomes and indicates that BCTV-Logan is more closely related to BCTV-California than to BCTV-CFH (Fig. 15). This was also suggested by earlier investigations of host range and symptom differences exhibited by these BCTV strains, and by restriction analysis of their genomes (Stenger et al., 1990). A comparison of the California and Logan strain coding sequences indicates that the proteins encoded by the two genomes are nearly identical. Previous comparison of the ORFs of the CFH and California strains indicated that the rightward reading frames retain over 95% conserved amino acid homology, whereas homology for the leftward ORFs varied from 86.7% for ORF L3
FIGURE 14. Nucleotide sequence of BCTV-Logan genome shown as the virion (+) sense. Nucleotide 1 is designated as the first nucleotide upstream of the ORF L1 start codon. The hairpin-loop motif conserved in geminiviruses (coordinates 83-114), and the triple repeat sequence discussed in the text (coordinates 288-395), are underlined.
FIGURE 15. Comparison of DNA sequence relatedness between the CFH (A) and California (B) strains and the Logan strain of BCTV. Plot denotes results of DNA homology matrix analysis using a window size of 20 nucleotides with a maximum number of five mismatches per nucleotide window. Nucleotide coordinates refer to the virion sense sequence and are listed on the axes.
"FIGURE 15 (continued),"
to as low as 56.7% for ORF L4 (D. C. Stenger, personal communication). These observations are also reflected in the DNA homology matrix (Fig. 15).

Located within the intergenic region is the sequence TAATATTAC flanked by inverted repeats of 10 nucleotides (nucleotide coordinates 83-114). This stem-loop motif is highly conserved among geminiviruses (Lazarowitz, 1987), is essential for replication (Revington et al., 1989), and is adjacent to the region which is bound by AL1 protein (Fontes et al., 1992; Thommes et al., 1993). Interestingly, all three strains exhibit sequence differences with respect to direct repeat elements downstream of this motif. Within the IR of the California strain is a 27 nucleotide direct repeat which has been suggested to resemble the 72 nucleotide repeat transcriptional enhancer element of SV40 (Stanley et al., 1986). Both the Logan and CFH strains retain only a single copy of a similar sequence element (nucleotide coordinates 184-210 in the Logan sequence). Downstream of this region the Logan sequence has a 36 nucleotide, nearly identical triple repeat (coordinates 288-395) which is absent in the other two strains. In order to investigate the importance of this triple repeat, plasmids pCT17 and pCT20, which contain 1.5 copies of tandemly repeated genomes of Logan containing only a single copy of the 36 nucleotide repeat element, were analyzed in protoplasts and plants, respectively. BCTV genomes containing only a single copy of the 36 nucleotide repeat element replicated in protoplasts at a level comparable to wild-type virus (Fig. 16). Also, this mutant infected plants with a virulence comparable to wild-type virus (Fig. 16). Since deletion of two of the 36 nucleotide repeat elements does not affect virulence of the Logan strain, and since the CFH strain retains only a single copy of each of the two types of repeat elements, it is probable that any potential function of these elements is retained when the elements are present on the genome in a single copy.
FIGURE 16. The 36 nucleotide triple repeat is not required for replication and infectivity. Three μg of DNA isolated from an uninoculated (H), a pCT6 inoculated (WT), and ten pCT20 inoculated N. benthamiana plants were electrophoresed on a 1% agarose gel and analyzed by Southern blotting. Also shown are the results of a Southern blot experiment where DNA (1 μg) isolated from protoplasts transfected with pCT2 and pCT17 was subjected to the Southern blotting procedure. Probes prepared from pCT9 and pCT10 were used for detection of BCTV DNA. The positions of open circular (OC), linear (LIN), supercoiled (SC), and single-stranded (SS) forms of genomic BCTV DNA, and of the inoculum open circular (Inoculum OC) and supercoiled (Inoculum SC) DNAs, are indicated.
Organization of ORFs and comparison of these with other geminivirus proteins. Analysis of the sequence of the California strain indicated that the BCTV genome is unique since the complementary sense ORFs are similar in size, location and sequence with those of the whitefly-transmitted geminiviruses, yet the only virion sense ORF which has a counterpart on the genome of these viruses is the coat protein (ORF R1). In addition, BCTV ORF R1 is more closely related to the coat protein of monopartite, monocot-infecting geminiviruses (Fig. 1, Table 1). These observations are also true of the Logan strain (Fig. 17) and are reflected in the similarity scores obtained when BCTV gene products are compared to their putative counterparts in other geminiviruses (Table 3).

Similarity scores based upon a protein weight matrix, such as the Dayhoff PAM 250, indicate the degree of similarity between proteins. The L1, L2, L3, and L4 BCTV proteins were compared with the corresponding leftward ORFs located on subgroup III geminiviruses, whereas the R2 and R3 proteins were compared with the precoat proteins of subgroup I geminiviruses. The coat protein of BCTV was compared with the coat proteins of all the geminiviruses used in this study. These scores are presented in Table 3. The similarity scores indicate that the complementary sense reading frames of BCTV are related to those of the bipartite subgroup III geminiviruses. For an adequate evaluation of these scores, it is relevant to note that scores of 59.9, 44.2, 44.7, and 30.6 are obtained when the AL1, AL2, AL3, and AL4 of TGMV and ACMV are compared by this method. An observation of some significance (to be discussed also in a later chapter), is that L2 is the least conserved of the four leftward reading frames. Although the similarity score for this protein indicates that it is derived from the analogous AL2 ORF of the bipartite geminiviruses, the constraint to maintain this protein has apparently been slight.
FIGURE 17. Arrangement of BCTV ORFs on the genome. The circular BCTV genome is shown as the dsDNA replicative form intermediate. ORFs are represented by arrows; leftward oriented arrows represent ORFs located on the complementary strand, whereas rightward arrows represent ORFs located on the virion sense strand. Indicated in tabular form to the right are the start and stop coordinates of the ORFs and the molecular weight of the proteins predicted from the sequence.
TABLE 3

BCTV PROTEINS AND COMPARISON WITH COUNTERPARTS IN SIX GEMINIVIRUSES

<table>
<thead>
<tr>
<th>Protein MW (kD)</th>
<th>ACMV</th>
<th>BGMV</th>
<th>TGMV</th>
<th>MSV</th>
<th>TobYDV</th>
<th>WDV</th>
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<tr>
<td>L1 40.6</td>
<td>58.4</td>
<td>68.6</td>
<td>65.9</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>L2 20.0</td>
<td>18.5</td>
<td>17.4</td>
<td>20.2</td>
<td>-</td>
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<td>39.5</td>
<td>58.8</td>
<td>65.9</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>R1 29.6</td>
<td>15.4</td>
<td>10.0</td>
<td>13.4</td>
<td>18.9</td>
<td>19.3</td>
<td>18.5</td>
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<tr>
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<tr>
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</tbody>
</table>

aNucleotide sequence of BCTV-CFH and BCTV-Worland (Dr. D. C. Stengers personal communication), and mutational analysis of this gene (Stanley et al., 1992) indicates that the functional gene product is initiated from an internal methionine residue, downstream of the first in-frame initiation codon. This shorter ORF is conserved in subgroup III viruses and has been used for comparison. As indicated in the text, subgroup I viruses lack L2, L3, and L4 ORFs.
The rightward genes of the subgroup I viruses are less conserved. Thus, for the three members used in this study, scores of 41.1 and 29.5 (for WDV vs TobYDV), 23.8 and 31.6 (for MSV vs TobYDV), and 24.4 and 28.7 (for MSV vs WDV) were obtained when the precoat (R2) and coat (R1) proteins, respectively, were compared. Unlike the subgroup I viruses, BCTV has two precoat reading frames (R1 and R2) and it is not apparent which of the two corresponds to the R2 ORF in the leafhopper-transmitted geminiviruses. However, the similarity scores indicate that neither R2 nor R3 are similar to the precoat proteins of the subgroup I viruses (Table 3). On the other hand, R1 shows a slightly greater similarity to the coat proteins of these viruses than it does to the coat proteins of subgroup III viruses. Since coat proteins are determinants of insect vector specificity (Briddon et al., 1990), and since BCTV and subgroup I viruses are transmitted by leafhoppers, this result is not unexpected.

The studies described in this chapter emphasize the hybrid nature of the BCTV genome. The organization of ORFs on this genome is unique since the complementary sense ORFs are similar to those of the whitefly-transmitted geminiviruses, yet the only BCTV virion sense ORF which has a counterpart on these genomes is the coat protein (Fig. 1). However, the coat protein appears to be more closely related to the coat proteins of other leafhopper-transmitted geminiviruses and mutational analysis suggests that it too, like the coat protein of viruses such as MSV, is required for spread and symptom development (Briddon et al., 1989; Stanley et al., 1992a; Hormuzdi and Bisaro, 1993).

Observations similar to those made above have also been noted previously for the California isolate (Stanley et al., 1986). However, previous studies failed to identify the R3 ORF and therefore this protein was not incorporated in studies pertaining to homology searches. This protein is required for infectivity (Hormuzdi and Bisaro, 1993), and is highly conserved in four BCTV isolates (Stanley, 1986;
Hormuzdi, this chapter; D.C. Stenger, personal communication) for which the sequence is known. However, the similarity scores obtained for ORF R3 do not suggest an origin for this gene from within another geminivirus subgroup.

A discussion concerning the conservation of nucleotide and protein sequence, and the evolution of geminiviruses, is most useful when a correlation between sequence and functional conservation is made. The studies described in the next two chapters describe studies designed to determine the function of BCTV gene products.
CHAPTER III

GENETIC ANALYSIS OF BEET CURLY TOP VIRUS: EVIDENCE FOR THREE VIRION SENSE GENES INVOLVED IN MOVEMENT AND REGULATION OF SINGLE- AND DOUBLE-STRANDED DNA LEVELS.

INTRODUCTION

The genome of the Logan strain of BCTV has been cloned (Stenger et al., 1990) and analysis of the nucleotide sequence (Chapter II), which differs little from that of the California isolate (Stanley et al., 1986), has revealed the presence of seven open reading frames capable of encoding proteins larger than 10 kD (Fig. 17). Four of the ORFs are complementary sense (leftward ORFs L1, L2, L3 and L4), and three of the ORFs are virion sense (rightward ORFs R1, R2, and R3). ORF R1 encodes the capsid protein (Briddon et al., 1989; Briddon et al., 1990). One of the rightward ORFs, namely R3, has not been reported previously although it is present in the California isolate. This ORF, which is large enough to encode a 10.0 kD protein, overlaps ORF R2 in a different reading frame. Evidence for the existence of three virion sense genes on a single genome component has not been presented for any geminivirus.

The complementary sense BCTV ORFs are homologous with respect to sequence and function to the complementary sense ORFs of bipartite geminivirus A components (Stanley et al., 1986; Stanley et al., 1992a). In this regard, BCTV is
easily distinguished from the monopartite members of subgroup I, which typically lack L2 and L3 ORFs and instead have two complementary sense ORFs which are fused by splicing to produce a single reading frame homologous to L1 (Schalk et al., 1989). On the other hand, the BCTV genome resembles the viruses of subgroup I in having multiple virion sense ORFs. The genomes of subgroup I geminiviruses without exception contain a coat protein ORF as well as a "precoat" ORF that have both been implicated in virus movement (Boulton et al., 1989; Lazarowitz et al., 1989; Boulton et al., 1991). In general, precoat ORFs are not highly conserved and so it is not clear if the BCTV R2 and R3 ORFs are related to the precoat ORFs of other monopartite viruses. Thus, the question arises as to the identity and number of virion sense BCTV genes that might be involved in movement, and whether a third virion sense gene exists in the BCTV genome and what its function might be.

In this chapter the results of mutagenesis of the rightward ORFs of BCTV are reported. Earlier observations of Briddon et al. (1989), who found that mutations in the coat protein gene render the virus non-infectious, are confirmed. Also, new information concerning the phenotypes of R2\(^{-}\) and R3\(^{-}\) mutants following inoculation of \(N.\) benthamiana plants and transfection of \(N.\) tabacum protoplasts is reported.

**MATERIALS AND METHODS**

**DNA techniques.** All restriction endonucleases and other DNA modifying enzymes were used as specified by the suppliers. Other techniques were performed essentially as described by Ausubel et al. (1987).
Amino acids in ORF

Wild type  Mutant
Protein     Protein

<table>
<thead>
<tr>
<th></th>
<th>R1</th>
<th>R2</th>
<th>L3</th>
<th>L2</th>
<th>L1</th>
<th>L4</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R1-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R1-Δ</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R2-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

254 156+3
254 179+0
102 67+6

Figure 18. Diagrams of BCTV rightward mutants. The 3038 bp circular genomic DNA of the Logan isolate of BCTV is shown in linear form, above which are boxes representing the ORFs. For any mutant genome, the ORF that is altered is indicated at the left. Restriction sites introduced or eliminated as a result of mutagenesis are indicated below the lines. The unshaded area within the R1-Δ genome, denoted by Δ, represents an in-frame deletion (amino acids 81-155) that is predicted to fuse the amino- and carboxy terminal ends of the coat protein. In other mutants, the unshaded portion of a box indicates a segment of protein derived from a different reading frame. To the right of the Figure, the number of amino acids in the wild-type and the mutant proteins is given. The latter is the total of amino acids from the original truncated ORF plus fused amino acids from different reading frames. The positions of restriction sites referred to in the text are indicated at the bottom of the figure. The first nucleotide adjacent to the initiation codon of ORF L1 is designated 1. ORF L4 spans nucleotides 3038/0; it has not been mutated in these studies.
"FIGURE 18 (continued),"

R2/3-

BamHI+ 525

R3-

BglII+ 463

Scal 52
BstXI 978
Csp45I 1205
DraI 1607
BglII 2337
SalI 2724

102 2+61
88 27+15
88 7+35

3038/0
Construction of BCTV mutants. BCTV wild-type and mutant genomes are illustrated in Fig. 18. A description of the manner in which the mutations were prepared follows. The construction of plasmids pCT3 and pCT4 has been described previously (Chapter II). Briefly, these plasmids contain 0.5 and 1.5 copies of the Logan genome respectively in pIC20H.

The R1' mutation was constructed by cleaving pCT4 with Csp45I at nucleotide 1205, followed by end-filling with the Klenow fragment of DNA polymerase I and re-ligation. This resulted in a 2 bp insertion which creates a unique NruI site and a frameshift within the R1 ORF. The construct was designated pCT14.

Plasmid pCT4 was restricted with Csp45I and BstXI followed by end-filling and ligation to generate mutation R1'-Δ, a 225 bp deletion mutant. The mutation is expected to produce a truncated R1 protein with an internal, in-frame deletion of 75 amino acids. The construct was designated pCT16.

Mutation R2' was created using the oligonucleotide 5'-GGTTAATTTTCTAGAAGTGTTCG-3' (beginning at nucleotide 713), which generates a 2 bp deletion and introduces a frameshift mutation and a unique XbaI site (underlined) into ORF R2. Mutation R3' was constructed using the oligonucleotide 5'-GGTCTGTCTACCTACAGATCTGG-3' (beginning at nucleotide 451), which results in the insertion of a single residue that introduces a frameshift and a BglII site (underlined) into ORF R3. Mutation R2/3' was constructed using the oligonucleotide 5'-GGGATCCCTTCCTAGAGTGATCGTC-3' (beginning at nucleotide 524), which inserts a single base pair and introduces a frameshift into both ORFs R2 and R3 as well as a new BamHI site (underlined). Site-directed mutagenesis (Kunkel et al., 1987) was performed using ssDNA obtained from pCT9, a pBluescript SK+ -based plasmid (Stratagene) containing the BCTV genome inserted at the Sall site. The resulting plasmids were designated pCT21 (R2/3'-), pCT24 (R3'-), and pCT37 (R2-). Plasmids
containing 1.5 copies of the viral genome but only one copy of the region of interest were obtained by inserting the full-length, modified genomes (as SalI fragments) into pCT3 to give plasmids pCT22 (R2/3-), pCT25 (R3-), and pCT38 (R2-).

For each of the constructs in pIC20H, a HindIII to ClaI fragment containing the tandem BCTV 1.5 repeat was inserted into the Ti plasmid vector pMON521 (a derivative of pMON505; Rogers et al., 1987) (Rogers et al., 1987). The relevant pMON521 derivatives are: pCT6 (wild-type), pCT18 (R1-), pCT19 (R1-Δ), pCT45 (R2-), pCT23 (R2/3-), and pCT28 (R3-). These plasmids were mated into Agrobacterium tumefaciens and the resulting strains were used to agroinoculate plant tissue.

Wild-type and mutant genomes were transfected into protoplasts as complete tandem dimers inserted at the SalI site of the plasmid pBluescript SK+. The pBluescript derivatives are: pCT52 (wild-type), pCT53 (R1-Δ), pCT54 (R3-), pCT55 (R2/3-), and pCT56 (R2-).

Plant, protoplast, and leaf disc inoculation and analysis. N. benthamiana and B. vulgaris L. (sugar beet) plants were agroinoculated with A. tumefaciens harboring Ti plasmids containing 1.5 copies of mutant and wild-type BCTV DNAs (Grimsley et al., 1987; Briddon et al., 1989). The inoculum was introduced into plants by the pin-pricking procedure which is described in Chapter I. Infections were detected by screening plants for symptoms and by Southern blot hybridization of total DNA extracted 30-45 days post-inoculation. N. tabacum suspension cell protoplasts were prepared and transfected as previously described (Sunter et al., 1990; Brough et al., 1992). DNA was isolated from protoplasts (7 days post-transfection) and plants by the method of Mettler (1987) and quantified by spectrophotometry. Southern blot hybridization (Ausubel et al., 1987) was performed using 32p-labeled riboprobes
prepared from BCTV clones in pBluescript SK+. For this purpose the clones pCT9 and pCT10 (Chapter II), which contain the BCTV genome in opposite orientation, were used. Direct quantification of DNA on Southern blots was carried out using a radioanalytic imager (Betascope 603, Betagen).

**Western blot analysis.** Crude virus preparations were prepared from BCTV-inoculated *N. benthamiana* leaf discs as described (Sunter et al., 1990), except that the crude virus pellet obtained following centrifugation at 125,000 g was resuspended in 200 µl 10 mM trisodium citrate, 1 mM EDTA, pH 7.0. Aliquots of crude virus preparations were electrophoresed through 12% polyacrylamide gels under denaturing conditions (Laemmli, 1970). The Western blot procedure was carried out as described (Sunter et al., 1990), using a primary antibody raised against BCTV virions (a gift of Dr. J. E. Duffus) at a dilution of 1:100,000. BCTV coat protein isolated from insect (Sf9) cells was graciously provided by Marcos D. Hartitz.

**RESULTS**

**Infectivity of BCTV virion sense mutants.** Mutations were introduced into each of the rightward BCTV ORFs as illustrated in Figure 18 and described in Materials and Methods, and the wild-type and mutant viral genomes were inserted as tandem repeats into Ti plasmid vectors. Following agroinoculation of plants, unit-length viral genomes capable of generating a systemic infection are released from the tandem repeats by replication or recombination (Elmer et al., 1988b; Stenger et al., 1991).
## TABLE 4

**INFECTIVITY OF BCTV, AND BCTV R1, R2, AND R3 MUTANTS**

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Inoculum&lt;sup&gt;a&lt;/sup&gt;</th>
<th>No. of experiments</th>
<th>Symptomatic plants&lt;sup&gt;b&lt;/sup&gt;</th>
<th>No. of experiments</th>
<th>Symptomatic plants&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>pCT6</td>
<td>4</td>
<td>36/36</td>
<td>3</td>
<td>13/14</td>
</tr>
<tr>
<td>R1-</td>
<td>pCT18</td>
<td>4</td>
<td>0/28</td>
<td>2</td>
<td>0/8</td>
</tr>
<tr>
<td>R1-Δ</td>
<td>pCT19</td>
<td>4</td>
<td>0/22</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>R2-</td>
<td>pCT45</td>
<td>5</td>
<td>0/34</td>
<td>2</td>
<td>0/12</td>
</tr>
<tr>
<td>R2/3-</td>
<td>pCT23</td>
<td>7</td>
<td>0/38</td>
<td>1</td>
<td>0/6</td>
</tr>
<tr>
<td>R3-</td>
<td>pCT28</td>
<td>4</td>
<td>0/28</td>
<td>3</td>
<td>0/14</td>
</tr>
</tbody>
</table>

**Coinoculations in N. benthamiana**

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Inoculum&lt;sup&gt;a&lt;/sup&gt;</th>
<th>No. of experiments</th>
<th>Symptomatic plants&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>R1-Δ, R2-</td>
<td>pCT19+pCT45</td>
<td>2</td>
<td>10/10</td>
</tr>
<tr>
<td>R1-, R2/3-</td>
<td>pCT18+pCT23</td>
<td>1</td>
<td>4/8</td>
</tr>
<tr>
<td>R1-Δ, R2/3-</td>
<td>pCT19+pCT23</td>
<td>2</td>
<td>5/12</td>
</tr>
<tr>
<td>R1-Δ, R3-</td>
<td>pCT19+pCT28</td>
<td>2</td>
<td>9/12</td>
</tr>
<tr>
<td>R2-, R3-</td>
<td>pCT45+pCT28</td>
<td>2</td>
<td>13/16</td>
</tr>
<tr>
<td>R2/3-, R3-</td>
<td>pCT23+pCT28</td>
<td>1</td>
<td>0/6</td>
</tr>
</tbody>
</table>

<sup>a</sup>Plants were inoculated with *A. tumefaciens* cells carrying Ti plasmid vectors containing 1.5 tandem repeats of the BCTV genome. Plants were scored for BCTV symptoms after 6 weeks.

<sup>b</sup>Number of plants showing symptoms/number of inoculated plants.

ND, not determined.
FIGURE 19. Viral DNA in plants agroinoculated with the R3- mutant. A Southern blot of DNA isolated from individual *N. benthamiana* plants inoculated with pCT28 (R3-, lanes 3-12) or pCT6 (wild-type, lanes 1 and 2) is shown. Each lane contains 5 μg of DNA, except lane 2 which contains 1 μg of the same DNA loaded in lane 1. The DNA was electrophoresed on a 1% agarose gel, transferred to Nytran membrane and hybridized with $^{32}$P-labeled riboprobes prepared using complementary sense and virion sense BCTV DNA as template. The left and right panels represent different exposures of the same membrane; the panel on the right was exposed longer than the panel on the left. The positions of unit-length open circular (OC), linear (LIN), and supercoiled (SC) dsDNA, unit-length ssDNA (SS), and various forms of subgenomic DNA (SG) are indicated.
FIGURE 20. Viral DNA in plants agroinoculated with the R2⁻ mutant. A Southern blot of DNA isolated from individual *N. benthamiana* plants inoculated with pCT45 (R2⁻, lanes 2-10) or pCT6 (wild-type, lanes 1 and 11) is shown. Lane 1 contains 4 µg, lane 11 contains 1 µg, and lanes 2-10 contain 2 µg of DNA isolated from systemically infected leaves. The DNA was electrophoresed on a 1% agarose gel, transferred to Nytran membrane and hybridized with ³²P-labeled riboprobes made using complementary sense and virion sense BCTV DNA as template. Viral DNA forms are indicated as in Figure 19.
All *N. benthamiana* and *B. vulgaris* L. (sugarbeet) plants inoculated with BCTV R1\(^{-}\), R1\(^{-}\Delta\), R2\(^{-}\), R2/3\(^{-}\) and R3\(^{-}\) mutants remained asymptomatic (Table 4). To determine whether the asymptomatic plants contained replicating viral DNA, total DNA was isolated from tissues distant from the inoculation site and subjected to Southern blot hybridization analysis. All inoculated plants (R1\(^{-}\), R2\(^{-}\), and R2/3\(^{-}\) mutants, six plants each examined; R3\(^{-}\) 10 plants examined) and *N. benthamiana* plants inoculated with R1\(^{-}\) and R1\(^{-}\Delta\) mutants (34 plants examined) and the R2/3\(^{-}\) double mutant (24 plants examined) did not contain detectable viral DNA (data not shown). However, some of the *N. benthamiana* plants inoculated with the R3\(^{-}\) mutant contained viral DNA (5 of 15 plants examined) but in much reduced amounts (less than 1% to 6%) relative to wild-type virus, as determined by radioanalytic analysis of the blot signals. A Southern blot of DNA isolated from 10 of these plants is presented in Figure 19. In contrast, almost all of the *N. benthamiana* plants inoculated with the R2\(^{-}\) mutant contained replicating BCTV DNA (11 of 12 plants examined). A Southern blot of DNA isolated from 9 of these plants is presented in Figure 20. Quantification of blot signals showed that viral dsDNA levels were similar in these symptomless plants to dsDNA levels in plants infected with wild-type virus, but ssDNA, which was visible upon longer exposure of the autoradiograph, was reduced by 80 to 100-fold. Subgenomic DNA species, which are produced in large amounts by wild-type BCTV also were greatly reduced and visible only upon longer exposure of the autoradiograph. Southern blot analysis of DNA isolated from sugarbeet plants inoculated with R1\(^{-}\), R2\(^{-}\), R2/3\(^{-}\), and R3\(^{-}\) mutants were subjected to Southern blot analysis. The data (not shown) indicate that the plants did not contain BCTV DNA in tissue distant from the inoculation site.

DNA extracted from *N. benthamiana* plants inoculated with wild-type BCTV or the R2\(^{-}\) mutant was treated with S1 nuclease or restricted with *Xba*I and *Sal*I and
FIGURE 21. Analysis of DNA from plants inoculated with R2- mutants. 2 μg of DNA isolated from an R2- inoculated plant (lanes 1-4), and from a wild-type BCTV inoculated plant (lanes 5-8) were either treated with S1 nuclease (lanes 2 and 6), digested with XbaI (lanes 3 and 7) or were digested with XbaI and SalI (lanes 4 and 8). Untreated samples are shown in lanes 1 and 5. Samples were subjected to Southern blot analysis as previously described. The positions of various BCTV DNA forms are indicated as before. Also shown are the positions where 1, 2, and 3 kb DNA fragments are expected to migrate.
analyzed on Southern blots (Fig. 21). Resistance to S1 nuclease (Fig. 21, lane 2) confirmed that the major BCTV-specific band visible in DNA from plants inoculated with the R2<sup>-</sup> mutant was dsDNA. Further, restriction endonuclease digestion conducted either with XbaI alone (Fig. 21, lane 3), or with XbaI and SalI (Fig. 21, lane 4), resulted in fragments consistent with the interpretation that this replicating BCTV dsDNA retained the unique XbaI site introduced by mutagenesis. As expected, wild-type BCTV DNA, which lacks an XbaI recognition sequence (Fig. 21, lane 7), was linearized when digested with XbaI and SalI (Fig. 21, lane 8).

**Virion sense mutants complement one another.** BCTV mutants were coinoculated onto *N. benthamiana* plants in pairwise combinations. All combinations, with the exception of the R3<sup>-</sup> + R2/3<sup>-</sup> pair, produced a systemic symptomatic infection (Table 4). In most cases, inoculated plants showed symptoms 2-3 weeks later than wild-type controls, which typically developed symptoms by 21 days post-inoculation. DNA from symptomatic plants was subjected to restriction endonuclease digestion and Southern blot analysis to determine whether the viral genomes retained their original mutations and if recombinant, wild-type genomes were present. For the combinations R1<sup>-</sup>A + R2<sup>-</sup> (3 plants tested) and R1<sup>-</sup>A + R3<sup>-</sup> (6 plants tested), some of the symptomatic plants lacked detectable amounts of recombinant wild-type DNA, whereas both mutant genomes were maintained (Fig. 22 and Table 5). In the case of the R1<sup>-</sup>A + R2<sup>-</sup> combination, the absence of wild-type recombinant DNA was indicated by the absence of a full-length linear (3038-bp) band following digestion with XbaI + ScaI and XbaI + Csp45I (Fig. 22B, lanes 4 and 5, respectively). For the R1<sup>-</sup>A + R3<sup>-</sup> combination, the absence of wild-type recombinant was indicated by the absence of a 3038 bp band following digestion with BglIII, the absence of a 2285 bp band upon digestion with BglII + ScaI, and the absence of a
### Table 5

**Restriction Fragments Generated by Restriction Endonuclease Digestion of Mutant BCTV DNAs**

<table>
<thead>
<tr>
<th>Enzyme&lt;sup&gt;a&lt;/sup&gt;</th>
<th>R1&lt;sup&gt;-Δ&lt;/sup&gt;</th>
<th>R2&lt;sup&gt;-&lt;/sup&gt;</th>
<th>R3&lt;sup&gt;-&lt;/sup&gt;</th>
<th>Wild-type</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>ScaI</em></td>
<td>2813</td>
<td>3036</td>
<td>3039</td>
<td>3038</td>
</tr>
<tr>
<td><em>Csp45I</em></td>
<td>uncut</td>
<td>3036</td>
<td>3039</td>
<td>3038</td>
</tr>
<tr>
<td><em>BglII</em></td>
<td>2813</td>
<td>3036</td>
<td>1875, 1164</td>
<td>3038</td>
</tr>
<tr>
<td><em>BglII</em> + <em>ScaI</em></td>
<td>2060, 753</td>
<td>n.r.&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1875, 753, 411</td>
<td>2285, 753</td>
</tr>
<tr>
<td><em>BglII</em> + <em>Csp45I</em></td>
<td>2813</td>
<td>n.r.</td>
<td>1164, 1132, 743</td>
<td>1906, 1132</td>
</tr>
<tr>
<td><em>XbaI</em></td>
<td>uncut</td>
<td>3036</td>
<td>uncut</td>
<td>uncut</td>
</tr>
<tr>
<td><em>XbaI</em> + <em>ScaI</em></td>
<td>2813</td>
<td>2366, 670</td>
<td>n.r.</td>
<td>3038</td>
</tr>
<tr>
<td><em>XbaI</em> + <em>BglII</em></td>
<td>n.r.</td>
<td>1613, 1423</td>
<td>1875, 1164</td>
<td>3038</td>
</tr>
<tr>
<td><em>XbaI</em> + <em>Csp45I</em></td>
<td>uncut</td>
<td>2555, 481</td>
<td>n.r.</td>
<td>3038</td>
</tr>
</tbody>
</table>

<sup>a</sup>The restriction enzymes listed are those used to generate the data presented in Figure 22.

<sup>b</sup>n.r., not relevant
FIGURE 22. Analysis of DNA from plants coinoculated with combinations of mutants. DNA was isolated from plants coinoculated with (A) R1Δ + R3-, (B) R1Δ + R2-, or (C) R2 + R3- mutants and analyzed by Southern blot hybridization using a probe specific for the complementary strand of BCTV DNA. This probe does not detect viral ssDNA. DNA in panel (A) was untreated (lane 1), or digested with ScaI (lane 2), Csp45I (lane 3), BglII (lane 4), BglII + ScaI (lane 5), and BglII + Csp45I (lane 6). DNA in panel (B) was untreated (lane 1), or digested with Csp45I (lane 2), ScaI (lane 3), XbaI + ScaI (lane 4), XbaI + Csp45I (lane 5), and XbaI (lane 6). DNA in panel (C) was untreated (lane 1), or digested with BglII (lane 2), XbaI (lane 3), and XbaI + BglII (lane 4). Size markers were generated by cleaving various plasmids containing BCTV DNA with a variety of restriction endonucleases; the positions of marker fragments (in kb) are given to the right of each panel. Uncut, supercoiled BCTV DNA migrated as a molecule of ~1.6 to 1.9 kb, as indicated in panel C. All samples contained a number of subgenomic DNAs of unknown size and conformation which complicates the identification of smaller fragments. The predicted sizes of restriction fragments generated from the full-length mutant DNAs are presented in Table 5.
"FIGURE 22 (continued)"
1906 bp band after digestion with *Bgl*II + *Csp*45I (Fig. 22A, lanes 4, 5, and 6, respectively). The apparent complementation observed with these mutant combinations suggests that the upstream ORFs encode transacting proteins that are functionally distinct from the coat protein, and that the lesions do not prevent the expression of coat protein. Similarly, in extracts from plants coinoculated with the R2⁻ + R3⁻ mutants, the bulk of the progeny viral genomes retained their original mutations, although a small amount of a DNA fragment (the 3038 bp fragment remaining after *Bgl*II + *Xba*I digestion) indicative of a recombinant genome was detected in each of the 9 plants tested (Fig. 22C, lane 4). That only a small fraction of the viral genomes in these plants appeared to be recombinant further supports the conclusion that ORFs R2 and R3 encode distinct transacting proteins.

**Replication of BCTV mutants in protoplasts.** Mutant BCTV genomes were inserted as tandem dimers into pBluescript vectors and transfected into protoplasts prepared from *N. tabacum* suspension culture cells in order to assess their ability to replicate unit-length genomic ssDNA and dsDNA. Replication was assayed by Southern blot hybridization analysis of total DNA isolated from protoplasts seven days post-transfection, followed by radioanalytic imaging to quantify replicated BCTV DNA forms. The averaged data from several experiments are presented in Table 6.

As shown in Figure 23, all of the mutants replicated DNA in protoplasts. Transfection of protoplasts with wild-type BCTV resulted in the appearance of viral DNA forms typically seen in infected plants, except that subgenomic forms were greatly reduced (Fig. 23, lanes 1 and 5). The R1⁻Δ, R2⁻, and R2/3⁻ mutants replicated the same unit-length DNA forms, however, in each case a decrease in ssDNA levels was observed (Fig. 23 lanes 2, 4, 6 and 7). The reduction in ssDNA levels seen with
<table>
<thead>
<tr>
<th>DNA form</th>
<th>R1-Δ</th>
<th></th>
<th></th>
<th>R2-</th>
<th></th>
<th></th>
<th>R2/3-</th>
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<th>R3-</th>
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<td>3</td>
<td>0.43-3.15</td>
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Note. Protoplasts were prepared from *N. tabacum* suspension cells and transfected with pBluescriptSK+ (Stratagene) based-plasmids containing tandem repeats of either wild-type or mutant genomes. DNA was isolated from protoplasts and subjected to Southern analysis. Quantification was carried out using a radioanalytic imaging system (Betascope 603). The values represent the amount of DNA detected in protoplasts transfected with BCTV mutants relative to wild-type DNA levels. Wild-type levels were arbitrarily assigned a value of 1. Values used to calculate total DNA levels were adjusted to account for strandedness.
FIGURE 23. Analysis of DNA from transfected protoplasts. DNA was isolated from protoplasts transfected with wild-type BCTV (lanes 1 and 5), or BCTV mutants R1Δ (lane 2), R3Δ (lane 3), R2/3Δ (lanes 4 and 6), and R2Δ (lane 7) and subjected to Southern blot analysis using 32P-labeled riboprobes specific for both virion and complementary sense BCTV DNA. Each lane contained 1 μg of total protoplast DNA. The positions of open circular (OC), linear (LIN), supercoiled (SC), and single-stranded (SS) BCTV DNA are indicated, as are the positions of open circular and supercoiled inoculum DNA.
R2* and R2/3' mutants was greater than that observed with the R1'Δ mutant, and was accompanied by an increase in dsDNA levels; on average seven times more than wild-type. As a consequence, total DNA levels attained by these mutants were slightly greater than wild-type BCTV (Table 6). The disruption of ORF R3 alone did not significantly affect the accumulation of either ssDNA or dsDNA (Fig. 23, lane 3), and the mutant appeared on average to replicate slightly better than wild-type virus (Table 6).

**Mutations in ORFs R2 and R3 do not prevent coat protein synthesis.** In order to directly determine whether the mutations in ORFs R2 and R3 prevent the expression of the downstream coat protein gene (R1), crude virus preparations obtained from leaf discs agroinoculated with wild-type and mutant viral genomes were probed on Western blots with antibody prepared against BCTV virions. Leaf discs were chosen for these experiments as relatively large amounts of material can be processed, facilitating the detection of accumulated coat protein.

In one experiment, aliquots of virus preparations containing approximately equal amounts of ssDNA were analyzed on Western blots, except for R1' and R1'Δ mutants where as much protein as possible was loaded. As many as five protein species were detected in crude virus preparations obtained from plants or leaf discs agroinoculated with wild-type BCTV (Fig. 24A, lanes 1 and 4). The two slowest migrating forms were present in reduced amounts in leaf disc extracts. The reason for the appearance of several coat protein bands is not known, but it is likely that most are specific degradation products. A doublet migrating at 29 kD was seen in extracts from insect cells infected with a recombinant baculovirus that expresses the R1 reading frame (Fig. 24A, lane 3). The two bands co-migrate with the slowest two of
FIGURE 24. Western blot analysis of crude virus preparations from tobacco leaf discs inoculated with BCTV mutants. Virus preparations were analyzed on Western blots probed with anti-BCTV antibody. Panel (A) shows leaf disc protein samples loaded on the basis of equivalent amounts of ssDNA (see text). Preparations from leaf discs inoculated with wild-type BCTV (0.4 µg, lane 4), and leaf discs inoculated with R2/3- (2.5 µg, lane 5), R3- (2.6 µg, lane 6), R1- (3.7 µg, lane 7), and R1-Δ (4.5 µg, lane 8) mutants are shown. Also shown is a virus preparation from infected plants (lane 1), a preparation from mock inoculated plants (lane 2), and a total protein extract from insect (SF9) cells infected with a recombinant baculovirus expressing the R1 ORF (lane 3). Panel (B) shows leaf disc samples loaded on the basis of equivalent amounts of protein (2.6 µg each). Preparations from leaf discs inoculated with wild-type BCTV (lane 3), and discs inoculated with R2/R3- (lane 5), R3- (lane 6), and R1- (lane 7) mutants are shown. Lane 1 contains a protein extract from SF9 cells expressing the R1 protein, lane 2 contains a virus preparation obtained from infected plants, and lane 4 contains a preparation from mock inoculated plants. The positions of 29 kD and 18.4 kD size markers are indicated.
the five bands seen in BCTV-infected plants and leaf discs. The faster migrating forms were also present in insect cell extracts, but in much smaller amounts.

The same BCTV-specific polypeptides observed in leaf discs inoculated with wild-type virus were detected at approximately the same intensity in virus preparations obtained from leaf discs inoculated with the R2/3- and R3- mutants (Fig. 24A, lanes 5 and 6). A similar pattern and intensity of BCTV-specific proteins also was observed in plants and leaf discs inoculated with the R2- mutant (data not shown). Thus, it appears that coat protein levels attained by wild-type BCTV and the R2-, R3- and R2/3- mutants are similar when ssDNA levels are taken into account, suggesting that these mutants produce a sufficient amount of coat protein to encapsidate the ssDNA present. As expected, extracts from mock inoculated plants (Fig. 24A, lane 2) or from leaf discs inoculated with coat protein mutants (Fig. 24A, lanes 7 and 8) did not contain protein that reacted specifically with the antibody.

In a second experiment, Western blot analysis was performed on aliquots representing equal amounts of protein from the crude virus preparations. As shown in Figure 24B, the same BCTV-specific polypeptides were detected under these conditions, although they were present in small amounts in leaf discs inoculated with R2/3- and R3- mutants. This result was not unexpected, and it can be argued that the reductions are attributable for the most part to effects of the mutations on movement efficiency and relative ssDNA levels rather than to direct effects on coat protein expression. Wild-type BCTV is able to efficiently spread within the inoculated leaf disc and is likely to infect a greater number of cells than R3- mutants, which appear to be defective in this process (discussed below). In addition, coat protein and ssDNA levels appear to some extent co-dependent, and R2- mutants accumulate reduced amounts of this DNA form. From these experiments, we conclude that mutations which disrupt ORFs R2 and R3 do not prevent the synthesis of coat protein. Further,
it seems unlikely that the differences in coat protein levels observed could alone account for the distinct behavior of R1\textsuperscript{-}, R2\textsuperscript{-} and R3\textsuperscript{-} mutants in plants and protoplasts.

DISCUSSION

The studies presented here describe the results of genetic analysis of the three virion sense ORFs of the Logan strain of BCTV. Our results confirm the previous observation of Briddon et al. (1989) that expression of the coat protein gene is required for systemic spread of the virus. Mutations in the R1 ORF abolished infectivity in *N. benthamiana* but did not prevent replication in *N. tabacum* protoplasts. A reduction in ssDNA levels relative to wild-type virus was observed, however, and similar or greater reductions in ssDNA levels have been noted previously with coat protein mutants of the California isolate of BCTV (Briddon et al., 1989), TGMV, and other geminiviruses (Sunter et al., 1990; Boulton et al., 1993).

We found that disruption of ORF R3 greatly reduced infectivity of the virus in *N. benthamiana*. The small proportion of plants that were infected remained asymptomatic and accumulated viral DNA in much reduced amounts, reminiscent of DNA A alone infections elicited by ACMV, AbMV, and TGMV in this host (Klinkenberg and Stanley, 1990; Evans and Jeske, 1993; S.G. Hormuzdi and D.M. Bisaro, Chapter II). The R3 mutation studied had no appreciable effect on viral DNA synthesis in protoplasts, nor did it prevent the synthesis of coat protein in leaf discs. It bears emphasizing that the R3\textsuperscript{-} mutant can be distinguished from coat protein mutants in protoplasts; in transient replication assays coat protein mutants accumulated reduced amounts of ssDNA, presumably because of the absence of coat protein, but the R3\textsuperscript{-} mutant did not. Thus, if the R3 lesion had a significant effect on coat protein expression it was not evident in the protoplast system. These
observations, and the apparent complementation of R1\(^{-}\) and R3\(^{-}\) mutants, suggest that the reduced infectivity of the R3\(^{-}\) mutant was not the result of a replication defect or a polar effect on the expression of coat protein.

The phenotype of wild-type replication in protoplasts but reduced infectivity in plants strongly suggests a direct role for the R3 gene product in systemic spread, and invites comparison of the movement systems of BCTV and subgroup I geminiviruses such as MSV. MSV also requires two rightward gene products, the coat protein and a smaller 11 kDa protein, for spread in the infected plant (Boulton et al., 1989; Lazarowitz et al., 1989; Boulton et al., 1993). However, the amino acid sequences of the MSV protein and the 10 kD R3 gene product are not conserved. Furthermore, the R3 gene product lacks a 25 amino acid hydrophobic \(\beta\)-sheet domain which is found in the precoat ORFs of TobYDV and the monocot-infecting geminiviruses (Boulton et al., 1993). Thus, it remains to be seen if any functional homology exists between the precoat ORFs of subgroup I geminiviruses and the R3 ORF. It should also be noted that the R3 ORF does not appear to share significant homology with the precoat ORFs found in the genomes of certain dicot-infecting, whitefly-transmitted geminiviruses such as ACMV and tomato yellow leaf curl virus (Navot et al., 1991; Kheyr-Pour et al., 1992).

The R2\(^{-}\) mutant was nearly as infectious as wild-type BCTV in \(N.\) benthamiana although the infections were asymptomatic. Therefore, in this host, R2 protein appears to be a symptom determinant in the sense that the absence of R2 function uncouples virus replication and spread from disease development. It is not possible to determine from the experiments performed whether R2 protein itself is responsible for eliciting disease symptoms or whether the lack of symptoms is a consequence of reduced accumulation of ssDNA. However, a correlation between symptom severity and ssDNA levels has been established previously in studies with
various TGMV and ACMV mutants (Gardiner et al., 1988; Sunter et al., 1990; Etessami et al., 1991). The data presented for R2* infections represent a more extreme example of this correlation, since in the asymptomatic plants dsDNA accumulated to wild-type levels, whereas ssDNA levels were reduced up to 100 fold. A phenotype suggesting a direct relationship between ssDNA and dsDNA levels was observed following transfection of protoplasts with the R2* and R2/3* mutants; on average, a nine-fold reduction in ssDNA levels was accompanied by about an eight-fold increase in dsDNA levels. Disruption of the R2 ORF did not prevent the expression of coat protein in leaf discs but a reduction in coat protein levels, most likely a result of reduced ssDNA levels and impaired movement, was observed.

It is curious that R2* mutants are not able to infect sugar beets, a natural host of BCTV. This result has also been noted previously (Stanley et al., 1992a). It is possible that this differential infectivity reflects less stringent requirements for virus replication and movement in *N. benthamiana* than in the natural host. Whether this is so remains to be seen, but in any event it will be prudent to include natural hosts in future studies whenever possible. On the other hand, the utility of *N. benthamiana* as a laboratory host is not diminished as a result of its "permissive" properties. Clearly, the R2* and R3* mutant studies reported here would have been less informative had they been limited to sugar beets.

That the R2* mutant can efficiently infect *N. benthamiana* plants argues against a direct role for the R2 gene product in virus movement, although decreased levels of ssDNA may contribute to a reduced ability of the virus to spread through the host. This would explain the reduction in ssDNA and dsDNA levels in plants infected with the R2* mutant, relative to protoplasts transfected with the same mutant. In addition to a possible indirect effect on movement, the altered ratio of ssDNA to dsDNA seen when BCTV genomes disrupted in R2 are introduced in protoplasts and
plants suggests that the R2 protein is involved in the regulation of viral DNA synthesis. The geminiviruses are believed to replicate their DNA by a rolling circle mechanism (Saunders et al., 1991; Stenger et al., 1991; Koonin et al., 1992) and a model for geminivirus DNA replication, based on the mechanisms of ssDNA containing coliphages (Komberg et al., 1992), would divide the process into three stages: SS→RF synthesis, RF→RF synthesis, and RF→SS synthesis. It is possible that the R2 gene product plays a direct role in the switch from RF→RF synthesis to RF→SS synthesis, so that the absence of R2 protein results in over-amplification of RF at the expense of ssDNA. The molecular basis of this phenotype is unclear, but perhaps an interaction between R2 protein and ssDNA or the replication complex in some way prevents complementary strand synthesis. This may occur in one of the two ways exemplified by the M13 gene 5 and φX174 gene C products (see Introduction chapter for further discussion). Alternatively, R2 protein may be involved in virus assembly. In this case, encapsidation might be the principal mechanism by which ssDNA is removed from the replication pool. The distinctly different phenotypes of coat protein and R2* mutants make this latter mechanism less attractive; while coat protein mutants do accumulate less ssDNA than wild-type virus, the reduction is not as pronounced as it is in R2* mutants. Coat protein mutants also do not over-accumulate dsDNA. Clearly, more work will be needed to elucidate the function of R2 protein, and the results of these studies may yield new insight into geminivirus replication mechanisms and plant DNA replication in general.

In a recent study (Stanley et al., 1992a), it was reported that mutations in BCTV (California strain) ORF V2 (equivalent to ORF R2) resulted in a symptomless infection in *N. benthamiana* associated with low levels of virus and decreased levels of ssDNA. One of the mutants examined in this study, ΔV2, displayed a phenotype identical to the R2* mutant. However, two other mutants, ΔV2+4 and ΔV2+14,
appeared to be non-infectious. This inconsistency was attributed to a polar disruption of coat protein expression by \( \Delta V2+4 \) and \( \Delta V2+14 \), although this was not investigated. An alternative explanation is offered by the results of the studies conducted in this chapter. Inspection of the nucleotide sequence of the California strain indicates that the \( \Delta V2+4 \) and \( \Delta V2+14 \) lesions disrupt both ORF R2 as well as the C-terminus of ORF R3. Thus, the behavior of these mutants is consistent with the interpretation that they are in fact R2/R3' double mutants. In contrast, the \( \Delta V2 \) lesion affects only ORF R2. The C to T transition and the T to A transversion used to engineer a termination codon in the R2 ORF (Stanley et al., 1992a) do not alter a leucine codon (CTT to TTA) present in ORF R3 at this position. Thus, the results of Stanley and coworkers and the results reported here are entirely consistent once the R3 ORF is recognized.

In summary, several lines of evidence indicate that the rightward half of the BCTV genome encodes three distinct, transacting gene products. 1) The R1", R1'A, R2' and R3" mutants showed unique phenotypes when introduced into \( N. benthamiana \) plants and \( N. tabacum \) protoplasts. In addition, the R2/R3" double mutant displayed a combination of the R2" and R3" phenotypes, verifying the results obtained with individual mutants and excluding the possibility that additional lesions introduced during \textit{in vitro} mutagenesis may have been responsible for the observed phenotypes. 2) The R1'A, R2' and R3" mutants appeared to complement one another. 3) The R2", R2/R3", and R3" mutants did not prevent the synthesis of coat protein in leaf discs, nor did these mutants appear to have a large, direct effect on coat protein expression levels. This is the first report of three functional virion sense genes on a single geminivirus genome component. The role of the rightward gene products in the BCTV replication cycle is currently under investigation.
CHAPTER IV

GENETIC ANALYSIS OF BEET CURLY TOP VIRUS: ROLES OF L2 AND L3 PROTEINS DURING PATHOGENESIS

INTRODUCTION

The nucleotide sequences of three BCTV strains have been determined (Chapter II). The cumulative sequence information indicates that the BCTV genome has seven conserved ORFs (Fig. 17). The requirement and possible functions of the genes R1, R2, and R3, which are located on the virion sense strand have been discussed in the previous chapter. The results indicate that the R1 and R3 genes are needed for virus transport and symptom production and that the R2 gene in addition to being a symptom determinant is essential for maintaining a balance between ss- and dsDNA synthesis during the infection cycle.

In addition to the three rightward genes, the BCTV genome also encodes the complementary sense ORFs L1, L2, L3, and L4, which show varying degrees of similarity with the AL1, AL2, AL3, and AL4 proteins located on the genomes of bipartite geminiviruses belonging to subgroup III (Chapter II; Stanley, 1986). The roles of the complementary sense genes of the bipartite geminiviruses have been determined and these are summarized in Table 1. Studies conducted with subgroup III viruses have implicated the AL1 gene product in replication, the AL2 gene product in transactivation of coat protein and BR1 gene expression and the AL3 gene product
in enhancing replication levels in an as yet undetermined manner (Brough et al., 1988; Elmer et al., 1988a; Revington et al., 1989; Sunter et al., 1990; Etessami et al., 1991; Morris et al., 1991; Sunter and Bisaro, 1991; Fontes et al., 1992; Haley et al., 1992; Lazarowitz et al., 1992; Sunter and Bisaro, 1992).

The complementary sense genes of BCTV also have been subjected to genetic scrutiny (Briddon et al., 1989; Stanley et al., 1992a; Stanley et al., 1992b). The results of these studies indicate that the L1 protein, like AL1 protein, is essential for replication, and that the L4 gene is a symptom determinant which affects the hyperplastic response of the host to BCTV infection. Mutagenesis studies on L2 and L3 ORFs have led to surprising conclusions. It was observed that the virus tolerated disruptions in L2 and L3 ORFs individually, but was made avirulent when the same mutations were combined onto a single genome. It was postulated that there is some redundancy in the functions of the L2 and L3 proteins, a conclusion which is not expected from an analysis of the amino acid sequences of the proteins, or by studies of the corresponding gene products in the bipartite geminiviruses.

The requirement of the L2 and L3 gene products for infection was investigated and the results are presented in the following pages. The results argue that the BCTV L3 gene product is similar to the AL3 protein encoded by the A genome of the subgroup III viruses. The experiments fail to explain earlier results pertaining to the roles of L2 and L3 (Stanley et al., 1992a), but clearly demonstrate that genomes bearing simultaneous disruptions in ORFs L2 and L3 can generate symptomatic infections when inoculated onto *N. benthamiana* and sugarbeets.
MATERIALS AND METHODS

DNA techniques. All restriction endonucleases and other DNA modifying enzymes were used as specified by the suppliers. Other techniques were performed essentially as described by Ausubel et al. (1987).

Construction of BCTV mutants. BCTV wild-type and mutant genomes are illustrated in Figure 25. A brief description of the manner in which the mutations were prepared follows. The construction of plasmid pCT1, and of plasmids pCT2 and pCT8, which were used as the wild-type constructs for protoplast and plant experiments, respectively, was described in Chapter II. Briefly, the pCT2 plasmid was constructed by inserting a unit-length BCTV (Logan strain) genome into the Sall site of pCT1, which contains ~0.5 copies of the BCTV genome cloned as a Sall-DraI fragment in pIC20H. Plasmid pCT8 was constructed by cloning the 1.5 tandemly repeated copies of BCTV-Logan from pCT2 into the Ti plasmid vector pMON521 (a derivative of pMON505; Rogers et al., 1987).

Mutation L2-1 was created using the oligonucleotide 5'-GTGAAGTGTCA GTTACAGGG-3' (beginning at nucleotide 1950), which introduces a UGA stop codon into ORF L2. Mutation L2-2 was constructed using the oligonucleotide 5'-CATGGTGTACTAGTGGCAGTTAC-3' (beginning at nucleotide 1939), which results in a 4 bp deletion and introduces an SpeI site (underlined). Mutation L2-3 was generated using the oligonucleotide 5'-GGATTGCATAATATTATGCAGGGG-3' (beginning at nucleotide 2116), which introduces a UAA stop codon into ORF L2 as well as an SspI site (underlined). This oligonucleotide also introduces a silent mutation in the L1 protein since it substitutes an ATT codon with an ATA codon, both of which code for isoleucine. Site-directed mutagenesis (Kunkel et al., 1987)
Amino acids in ORF

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<th>Wild type Protein</th>
<th>Mutant Protein</th>
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</tr>
<tr>
<td>L2-1</td>
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<td>72</td>
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<tr>
<td>L2-3</td>
<td>173</td>
<td>13</td>
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</table>

**FIGURE 25.** Diagrams of BCTV leftward mutants. The 3038 bp circular genomic DNA of the Logan isolate of BCTV is shown in linear form, above which are boxes representing the ORFs. For any genome, the mutant that is represented is indicated at the left. Restriction sites introduced or eliminated as a result of mutagenesis are indicated below the lines. In mutants L2/3-1, L2/3-2, and L3, the dark segment of the ORF indicates a segment of protein derived from a different reading frame. To the right of the figure, the number of amino acids in the wild-type and the mutant proteins is given. The latter is the total of amino acids from the original truncated ORF plus fused amino acids from different reading frames. The positions of restriction sites referred to in the text are indicated at the bottom of the figure. The first nucleotide adjacent to the initiation codon of ORF L1 is designated 1. ORF L4 spans nucleotides 3038/0; it has not been mutated in these studies.
"FIGURE 25 (continued),"

- L2/3-1
  - BspEI/EagI+ 1767
  - NcoI 2499
  - SspI 2590

- L2/3-2
  - Dral/KpnI+ 1607
  - SpeI+ 1947

- L3
  - Dral/KpnI+ 1607

- L2/3-1:
  - BspEI/EagI+ 1767
  - 173 134 + 4
  - 137 58 + 40

- L2/3-2:
  - Dral/KpnI+ 1607
  - 173 72
  - 137 111 + 16

- L3:
  - Dral/KpnI+ 1607
  - 137 111 + 16

- SspI 97
- SpeI 826
- SspI 1508
- BspEI 1767
- NcoI 2499
- SalI 3038/0
- Csp45I 1205
- Dral 1607
- SspI 2590
was performed using ssDNA obtained from pCT9, a pBluescript SK+ -based plasmid (Stratagene) containing the BCTV genome inserted at the SalI site. The resulting plasmids were designated pCT26 (L2-1), pCT35 (L2-2), and pCT36 (L2-3).

An 8 bp (5'-GGGTACCC-3') insertion containing a unique KpnI site (underlined) was introduced in the middle of the unique DraI restriction site in the BCTV genome. This was accomplished by ligating together the HindIII -KpnI fragments from pCT1 and pCT3. The resulting construct, cloned in pIC20H and designated pCT65 (L3), harbors a full-length BCTV genome disrupted in ORF L3.

Plasmids containing 1.5 copies of the viral genome but only one copy of the region of interest were obtained by inserting the full-length, modified genomes (as SalI fragments) into pCT1 to give plasmids pCT27 (L2-1), pCT39 (L2-2), pCT42 (L2-3) and pCT83 (L3).

The L2/3-1 genome was constructed by cleaving pCT2 with BspEI at nucleotide 1767, followed by end-filling with the Klenow fragment of DNA polymerase I and re-ligation. This resulted in a 4 bp insertion which creates a unique EagI site and a frameshift within the L2 and L3 ORFs. The construct was designated pCT70. L2/3-2 was constructed by replacing the pCT83 (L3 mutant) NcoI (nucleotide coordinate 2499) -BspEI fragment with the corresponding fragment from pCT39 (L2-2 mutant). The resulting construct, which combines the individual L2 and L3 mutations from pCT39 and pCT83 into a single genome, was designated pCT95. The wild-type (WT*) construct obtained by the reciprocal replacement of the pCT39 NcoI-BspEI with the corresponding one from pCT83 was designated pCT96.

For each of the constructs in pIC20H, a HindIII to ClaI fragment containing the tandem BCTV 1.5 repeat was inserted into the Ti plasmid vector pMON521 (a derivative of pMON505; Rogers et al., 1987). The relevant pMON521 derivatives are: pCT8 (wild-type), pCT98 (WT*), pCT29 (L2-1), pCT44 (L2-2), pCT59 (L2-3),
pCT74 (L2/3), pCT97 (L2/3-2) and pCT90 (L3). These plasmids were mated into Agrobacterium tumefaciens and the resulting strains were used to agroinoculate plant tissue.

For complementation experiments, a construct designed to express only the L3 ORF (pCT63) was made by replacing the GUS coding sequence between the cauliflower mosaic virus 35S promoter and the nopaline synthase 3' end in pBI221 (Clonetech, Palo Alto, CA) with a 439 bp SpeI-SspI fragment derived from pCT35 (L2-2). The construction of the plasmids pTGA45, which contains a deletion in the TGMV AL3 ORF, and pTGA81, which is similar to pCT63 except that the construct contains the TGMV AL3 ORF, have been described previously (Sunter et al., 1990; Sunter et al., 1993).

Inoculation of plants. Healthy Nicotiana benthamiana plants at the six-leaf to eight-leaf stage were inoculated at the shoot tip with Agrobacterium cultures (agroinoculation; Grimsley et al., 1986; Grimsley and Bisaro, 1987). Shoot tips were excised with a scalpel and the inoculum was applied to the cut stem. The cut stem was then pricked multiple times with an insect pin. The inoculation procedure employed for sugarbeet plants was essentially the same except that the inoculum was applied to the intact apical region of the plant.

Protoplast transfection. Protoplasts were prepared from N. tabacum cv. Wisconsin suspension culture cells (a gift of Dr. D. P. S. Verma) and transfected essentially as described by Potrykus and Shillito (1986) and Negrutiu et al. (1987), with minor modifications (Brough et al., 1992; Inamdar et al., 1992).
DNA isolation and analysis. DNA from protoplasts and plants was isolated according to the procedure of Mettler (1987), and quantified by spectrophotometry. Samples containing DNA were electrophoresed through 1% agarose gel, transferred to Nytran membrane (Schleicher and Schuell, Keene, NH) and analyzed by hybridization with $^{32}$P-labeled riboprobes prepared from BCTV clones in pBluescript SK+ (Stratagene, La Jolla, CA) vectors. Direct quantification of DNA on Southern blots was carried out using a radioanalytic imager (Phosphorimager, Molecular Dynamics).

Statistical analysis of protoplast data. The total DNA values obtained after Southern blot hybridization and quantification of BCTV DNA forms using the Phosphorimager (Molecular, Dynamics) was subjected to a one way analysis of variance using the Minitab data analysis software (Minitab Incorporated, State College, Pa). Statistical analyses was done on three separate sets of data since the transfections were conducted at different times. Thus, experiments involving L2-1 and wild-type, L2-2, L2-3, and wild-type, and L2/3-1, L3, and wild-type were analyzed separately. Pooled standard deviation values of 0.2, 0.25, and 0.07 respectively were obtained for the three sets of data.

RESULTS

L2, L3, and L2/3 mutants are infectious. All of the leftward mutants produced symptoms in both *N. benthamiana* and sugarbeets (Table 7). The symptoms produced by these mutants were similar but not identical to wild-type BCTV (see below). Total DNA isolated from inoculated plants was subjected to Southern blot analysis and the results are presented in Figures 26, 27, and 31.
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<th>Mutation</th>
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<th>Sugarbeet Infectivity</th>
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<td></td>
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<td>Symptomatic plants&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>L3</td>
<td>pCT90</td>
<td>3</td>
<td>50/50</td>
</tr>
</tbody>
</table>

<sup>a</sup>Plants were inoculated with <i>A. tumefaciens</i> cells carrying Ti plasmid vectors containing 1.5 tandem repeats of the BCTV genome. Plants were scored for BCTV symptoms.

<sup>b</sup>Number of plants showing symptoms/number of inoculated plants. ND, not determined.
FIGURE 26. Viral DNA in sugarbeet plants agroinoculated with BCTV doubly mutated in the L2 and L3 ORFs. A Southern blot of DNA isolated from individual sugarbeet plants inoculated with wild-type (WT; 1 µg) BCTV or with the L2/3-1 mutant BCTV (5 µg) is shown. Total DNA was isolated from systemically infected leaves, electrophoresed on a 1% agarose gel, transferred to Nytran membrane and hybridized with $^{32}$P-labeled riboprobe made using complementary sense and virion sense BCTV DNA as template. The positions of unit-length open circular (OC), and supercoiled (SC) dsDNA, and unit-length ssDNA (SS) are indicated.
**FIGURE 27.** Viral DNA in sugarbeet plants agroinoculated with L2 and L3 ORF mutants. A Southern blot of DNA isolated from individual sugarbeet plants inoculated with wild-type (WT) BCTV or with the mutant BCTV genomes is shown. Each lane contains 2 µg of DNA isolated from systemically infected leaves. The DNA was electrophoresed on a 1% agarose gel, transferred to Nytran membrane and hybridized with ³²P-labeled riboprobes made using complementary sense and virion sense BCTV DNA as template. The positions of unit-length open circular (OC), linear (LIN), and supercoiled (SC) dsDNA, unit-length ssDNA (SS), and various forms of subgenomic DNA (SG) are indicated.
FIGURE 28. *N. benthamiana* plants inoculated with L2/3-2 mutant are infectious. The photograph shows a couple of *N. benthamiana* plants agroinoculated with the L2/3-2 mutant and showing typical BCTV symptoms.
Figure 29. Recovery of symptomatic *N. benthamiana* plants induced by L2/3-1. The upper photograph shows an uninoculated plant (MOCK) and of plants agroinoculated with wild-type (WT), and L2/3-1 BCTV constructs. The bottom photograph shows the recovered and symptomatic tissue produced by the L2/3-1 inoculated plant.
FIGURE 30. Differences in recovery induced by L3 mutant and wild-type BCTV. The photograph is taken of wild-type (WT) and L3 mutant inoculated *N. benthamiana* plants (two each) ~six weeks after the first symptoms of BCTV infection. Note the recovered tissue produced by one of the two plants inoculated with wild-type BCTV.
shown is a photograph taken of the recovered shoot. Nearly all plants inoculated with mutations in L2 and L3 ORFs show enhanced recovery. A noticeable exception is the L2-3 mutant which appeared to be wild-type with respect to symptom development and recovery (data not shown). Plants inoculated with L2-3 and wild-type BCTV also induced the production of recovered shoots, however, these shoots were produced in a smaller proportion of plants and were induced much later, i.e. about three weeks after the onset of recovery with L2 and L3 mutants. A photograph of plants inoculated at the same time with L3 and wild-type BCTV illustrates the difference in the recovery they induce (Fig. 30).

In order to determine whether recovered tissue contained replicating viral DNA, total DNA was extracted from recovered tissue from plants infected with the L2-2, L2-3 and L3 mutants and subjected to Southern blot analysis (Fig. 31). Total DNA was also isolated from L2-2 and L3 inoculated plant tissue early in the infection cycle, prior to the onset of recovery, and from L2-3 inoculated plants which were devoid of recovered tissue. These DNA samples were run on a 1% agarose gel and analyzed on Southern blots (Fig. 31). For most DNA samples isolated from recovered tissue, the amount of viral DNA produced were below detectable levels, whereas DNA from symptomatic tissue contained BCTV viral DNA forms in abundance (Fig. 31). The results demonstrate that the recovery phenotype is associated with a reduction in levels of viral DNA. This is also suggested from previous studies in which it was noted that virus concentration is reduced in recovered tissue (Bennett, 1971).

That BCTV mutants did not revert to a wild-type form was indicated by the maintenance of restriction sites used to generate the mutations. This was demonstrated for L2-2, L2/3-1, and L3. In each case, restriction digestion resulted in fragments consistent with the interpretation that the replicated virus retained the
FIGURE 31. Reduced levels of viral DNA in recovered tissue. A Southern blot of DNA isolated from individual *N. benthamiana* plants inoculated with wild-type (WT) BCTV or with the mutant BCTV genomes is shown. DNA from L2-2, L2-3, and L3 inoculated plants was isolated either from symptomatic tissue or from recovered tissue (R; four, two, and four samples respectively). Each lane contains 1 μg of DNA isolated from systemically infected leaves. The DNA was electrophoresed on a 1% agarose gel, transferred to Nytran membrane and hybridized with $^{32}$P-labeled riboprobes made using complementary sense and virion sense BCTV DNA as template. The positions of unit-length open circular (OC), linear (LIN), and supercoiled (SC) dsDNA, unit-length ssDNA (SS), and various forms of subgenomic DNA (SG) are indicated.
Figure 32. Analysis of DNA from plants inoculated with L2 and L3 mutant BCTV genomes. A. A Southern blot of DNA from sugar beet plants inoculated with wild-type (WT, 1 µg) or L2/3-1 (5 µg) and either untreated (UNCUT), or digested with BspEI or EagI is shown. B. DNA from N. benthamiana plants inoculated with wild-type (WT, 3 µg), L2-2 (2 µg), or L3 (3 µg) were either untreated (UNCUT) or were digested with Csp45I, SpeI or KpnI as indicated. Samples were subjected to Southern blot analysis as previously described and the positions of various BCTV DNA forms are indicated as before. Subgenomic forms of BCTV DNA migrate more rapidly than unit-length supercoiled (SC) DNA. The arrow indicates the predicted location of the ~1100 bp fragment obtained after restriction of the L2-2 genome with SpeI. It should be noted the visibility of this fragment is complicated by the appearance of several subgenomic fragments in that area of the gel.
mutation site. Thus, BCTV DNA from L2/3-1 and L3 inoculated plants produced unit-length fragments upon restriction with EagI and KpnI, respectively, and DNA from an L2-2 inoculated plant produced fragments of ~1100 and 1900 bp when restricted with SpeI. It should be noted that the 1900 bp fragment produced after SpeI restriction of L2-2 inoculated plant DNA co-migrates with supercoiled DNA under these conditions (also noted previously in Hormuzdi and Bisaro, 1993). However, the absence of open circular and linear DNA forms after restriction (compare uncut lane with SpeI restricted lane, also wild-type DNA restricted with SpeI), suggests that the DNA in the restricted sample which co-migrated with supercoiled BCTV DNA is the 1900 bp fragment. It should also be noted that the 1100 bp fragment produced after SpeI restriction of L2-2 inoculated plant DNA co-migrates with subgenomic forms of BCTV DNA (Fig. 32, predicted position of this fragment is indicated by the arrow). These results indicate that the mutant BCTV genomes were responsible for the production of symptoms in each case and, therefore, that the L2 and L3 genes are not required for systemic spread and symptom production.

Replication of L2 and L3 mutants in protoplasts. The replication efficiency of genomes bearing disruptions in the L2 and L3 reading frames was investigated by transfecting the mutants into protoplasts prepared from N. tabacum suspension cell cultures. The amount of ss- and dsDNA produced following each transfection was quantified using a radioanalytic imaging system (Phosphorimager, Molecular Dynamics). The averaged data from a number of experiments is shown in Table 8.

Typically, L2-1 and L2-2 mutants did not replicate as well as wild-type virus or the L2-3 mutant (Fig. 33). However, a statistical analysis of variance conducted on total DNA levels accumulated by L2-1, L2-2, and L2-3 mutants and wild-type virus
### TABLE 8

**RELATIVE REPLICATION LEVELS OF BCTV L2 AND L3 MUTANTS IN *Nicotiana tabacum* PROTOPLASTS**

<table>
<thead>
<tr>
<th></th>
<th>L2-1</th>
<th>L2-2</th>
<th>L2-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>dsDNA</td>
<td>3 0.32-0.99</td>
<td>0.66</td>
<td>5 0.35-1.03</td>
</tr>
<tr>
<td>ssDNA</td>
<td>3 0.44-0.96</td>
<td>0.63</td>
<td>5 0.45-1.38</td>
</tr>
<tr>
<td>Total DNA</td>
<td>3 0.40-0.93</td>
<td>0.73</td>
<td>5 0.50-1.26</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>L2/3-1</th>
<th>L2/3-2</th>
<th>L3</th>
</tr>
</thead>
<tbody>
<tr>
<td>dsDNA</td>
<td>7 0.29-0.37</td>
<td>0.33</td>
<td>1 -</td>
</tr>
<tr>
<td>ssDNA</td>
<td>7 0.06-0.47</td>
<td>0.23</td>
<td>1 -</td>
</tr>
<tr>
<td>Total DNA</td>
<td>7 0.15-0.44</td>
<td>0.27*</td>
<td>1 -</td>
</tr>
</tbody>
</table>

Note. Protoplasts were prepared from *N. tabacum* suspension cells and transfected with pBluescriptSK+ (Stratagene) based-plasmids containing tandem repeats of either wild-type or mutant genomes. DNA was isolated from protoplasts and subjected to Southern analysis. Quantification was carried out using a radioanalytic imaging system (Phosphorimager, Molecular Dynamics). The values represent the amount of DNA detected in protoplasts transfected with BCTV mutants relative to wild-type DNA levels. Wild-type levels were arbitrarily assigned a value of 1.

*An ANOVA statistical analysis was conducted for total DNA levels replicated by the mutants. For experiments involving L2/3-1, L3, and wild-type, a significant difference in the deviation of the means for the two mutants from wild-type mean (1.0) was obtained with a 99% confidence interval (standard deviation = 0.07).
FIGURE 33. Analysis of DNA from protoplasts transfected with mutations in the L2 and L3 ORFs. A Southern blot of DNA isolated from protoplasts transfected with wild-type BCTV (WT or WT*), or BCTV mutants L2-1, L2-2, L2-3, L2/3-1, and L2/3-2 is shown. The DNAs were subjected to Southern blot analysis using $^{32}$P-labeled riboprobes specific for virion sense BCTV DNA. Each lane contained 1 µg of total protoplast DNA except for the WT* and L2/3-2 lanes which contained 0.5 µg of protoplasts DNA. The positions of open circular (OC), linear (LIN), supercoiled (SC), and single-stranded (SS) BCTV DNA are indicated, as are the positions of open circular and supercoiled inoculum DNA.
failed to indicate a significant difference in their replication efficiencies. The reduction in levels of replication shown by genomes bearing disruptions in L3 were much greater, with the result that protoplasts transfected with L2/3-1, L2/3-2, and L3 mutants (Fig. 33) only accumulated 20-30% of wild-type total DNA levels. In the case of these mutants, a significant difference in replication levels compared to wild-type was observed (pooled standard deviation = 0.07; Table 8).

The L3 and AL3 proteins are functionally equivalent. A TGMV AL3 mutant was demonstrated to replicate ss- and dsDNA at levels 50-fold lower than wild-type virus (Sunter et al., 1990). Thus, the phenotype of BCTV L3 mutants is similar to the phenotype noted for AL3 mutants, although the reduction in replication levels in the case of BCTV is less severe. In order to determine whether the two gene products are functional counterparts, complementation experiments were undertaken. The objective of these experiments was to complement the AL3<sup>-</sup> phenotype with the BCTV L3 protein, and reciprocally, the L3<sup>-</sup> phenotype with the AL3 protein from TGMV. For this purpose, the L3 and AL3 reading frames were cloned downstream of the strong and constitutive CaMV 35S promoter and the resulting constructs were utilized in cotransfection experiments.

The results of these studies are shown in Figure 34. The ability of the construct containing the L3 ORF to produce a functional gene product was demonstrated by the replication of BCTV DNA to wild-type levels in protoplasts transfected with either the L3 or L2/3-1 mutants and the BCTV L3 expression construct (pCT63; Fig. 34). In the absence of the cotransfected L3 ORF, the levels of replicated DNA remained low. It was previously demonstrated that a construct expressing the TGMV AL3 ORF (pTGA81) is capable of complementing a construct containing 1.5 copies of the TGMV genome with a deletion in the AL3 ORF (Sunter
FIGURE 34. Complementation of L3- and AL3- phenotypes by the BCTV L3 and TGMV AL3 proteins. DNA was isolated from protoplasts transfected with the various BCTV and TGMV constructs as indicated and subjected to Southern blot analysis using 32P-labeled riboprobes specific for complementary sense BCTV DNA. Each lane contained 1 µg of total protoplast DNA except for the lanes located in the panel to the extreme right which contained 0.5 µg of DNA. The positions of open circular (OC), linear (LIN), supercoiled (SC), and single-stranded (SS) BCTV DNA are indicated.
et al., 1993). The L3 and AL3 expression constructs were found to be capable of reciprocal complementation (Fig. 34). When cotransfected with the TGMV AL3 mutant, the plasmid designed to express BCTV L3 protein was able to compensate for the deficiency in AL3 and restore wild-type levels of replicating TGMV DNA (Fig. 34). Similarly, TGMV AL3 protein was able to complement the reduced DNA phenotype associated with disruption of the BCTV L3 ORF. Complementation was not observed when a construct designed to express the BCTV R2 ORF was cotransfected with the TGMV mutant.

DISCUSSION

The experiments presented in this chapter concern the requirement of the L2 and L3 BCTV gene products for infectivity. Studies on the functions of the analogous reading frames present on the genomes of the bipartite, whitefly-transmitted geminiviruses have demonstrated that the AL2 gene product is required for the expression of the AR1 and BR1 genes (Sunter and Bisaro, 1991; Haley et al., 1992; Sunter and Bisaro, 1992). As a consequence, the disruption of ORF AL2 results in a lack of infectivity and in reduced accumulation of coat protein and ssDNA, which can be observed in protoplasts (Elmer et al., 1988a; Sunter et al., 1990; Etessami et al., 1991). The role of the AL3 protein is unknown but it has been shown to be necessary for enhancing replication levels and for the production of wild-type symptoms (Elmer et al., 1988a; Sunter et al., 1990; Etessami et al., 1991; Morris et al., 1991). Mutants bearing lesions in this reading frame display attenuated symptoms when inoculated on N. benthamiana plants.

Previous studies of BCTV (strain California) have suggested that the L2 and L3 gene products are redundant to some extent since it was shown that genomes
bearing disruptions in either one of these genes are infectious, whereas genomes disrupted in both these genes simultaneously are incapable of producing an infection (Stanley et al., 1992a). These results are surprising because there has been no indication of a similar functional redundancy in the bipartite geminiviruses and there is no obvious similarity in the amino acid sequence of the L2 and L3 proteins (a similarity score of 10.3 was obtained when the amino acid sequences of BCTV L2 and L3 proteins were compared using the SeqApp computer application). In the studies presented in this chapter three mutations in L2, one in L3, and two in both the L2 and L3 ORFs of BCTV-Logan were generated and analyzed. Particular care was taken in the construction of the double mutants: L2/3-1 generated a frameshift in both L2 and L3 reading frames by alteration of the genomic sequence at a single location, while L2/3-2 combined the altered sequences of individual L2 and L3 mutants. The lesions in the L2/3-2 mutant are similar to those constructed in BCTV-California (ΔC2/C3; Fig. 35; Stanley et al., 1992a). All of the Logan strain L2 and L3 mutants were able to infect sugarbeet and *N. benthamiana* plants. Thus, neither the L2 nor the L3 gene products alone are required for infectivity, a conclusion which was also reached in the previous study (Stanley et al., 1992a). However, Logan strain genomes disrupted in both the L2 and L3 ORFs were found to be as infectious as the single mutants. These results argue against the hypothesis that the L2 and L3 gene products have some redundant function or function cooperatively in BCTV infection. In this respect, the conclusions arrived at in this study differ from those reached by Stanley et al (1992a), and the reason for this discrepancy is unknown.

Although the mutants infected sugarbeets with a virulence equivalent to wild-type virus, the symptoms they produced on *N. benthamiana* were very different. Plants infected with L2-1, L2-2, L2/3-1, L2/3-2, and L3 mutants rapidly recovered from the early severe BCTV symptoms. A reduction in BCTV DNA levels was
**FIGURE 35.** Comparison of the BCTV-California and BCTV-Logan (ΔC2/C3 and L2/3-2) double mutants. Specific regions of the L2 (right side sequence) and L3 (left side sequence) genes are shown as virion strand DNA. The region between the L2 and L3 gene segments under consideration is represented by a broken line. The nucleotide sequence of wild-type California and Logan strain genomes, and of the ΔC2/C3 and L2/3-2 mutant genomes are shown. It should be noted that wild-type BCTV-California and BCTV-Logan are identical for the portion of the genome under consideration. Insertion sites in the L2/3-2 mutant are indicated by the arrows, below which are shown the inserted nucleotides. Deleted nucleotides in L2/3-2 mutant are shown by the symbol ‘Δ’. Nucleotide substitutions in the ΔC2/C3 mutant are underlined.
correlated with the attenuation of symptoms. This observation is also consistent with previous studies which noted a reduction in levels of virus particles in recovered tissue (Bennett, 1971). A recovery phenotype was also observed in plants inoculated with wild-type BCTV and the L2-3 mutant, however, the recovery phenotype was induced infrequently and generally appeared much later. One interpretation of these results is that both the L2 and L3 gene products can independently induce the enhanced recovery phenotype. Furthermore, since the L2 and L3 proteins are different and do not appear to encode a redundant function (see preceding paragraphs), it could be argued that L2 and L3 mutants induce recovery by distinct mechanisms. In this case the different behavior of the L2-3 mutant may be due to the fact that the mutation is located in a region of the L2 ORF which does not actually form part of the L2 protein. As shown in Figure 36, the BCTV L2 ORF is longer than its counterparts in the subgroup III viruses. However, if an internal methionine initiation codon is used, a reasonably good alignment between these proteins and BCTV L2 protein can be constructed. Thus, if the L2 protein initiates at the downstream methionine codon, the L2-3 mutant would be wild-type with respect to its ability to form a functional L2 protein (Fig. 36). However, it is possible that the BCTV L2 ORF appears as such only because almost all of its sequence is overlapped by the conserved L1 and L3 genes (Fig. 36). Thus, it is possible that only the L3 gene product is responsible for inducing recovery and that the L2-1 and L2-2 mutants affect expression of the downstream L3 gene. This scenario seems less plausible since the phenotype of L2-1 and L2-2 mutants in protoplasts does not indicate that they affect L3 gene expression. More detailed examination of L3 gene expression in these mutants, the determination of the nucleotide sequence of additional BCTV strains, and knowledge of the initiation sites of leftward transcripts may differentiate
between these two alternatives. At this time, however, we cannot unequivocally assign a function to the BCTV L2 ORF.

The phenomenon of recovery associated with BCTV infection has been studied extensively and all *Nicotiana* species tested show varying degrees of recovery (Bennett, 1971). Interestingly, this phenomenon has been likened to immunological reactions induced by animal viruses. Studies have indicated that recovered plants are resistant to reinfection with BCTV and that the recovery condition can be passed on to healthy plants by scions from recovered plants (reviewed in Bennett, 1971). It was previously suggested that recovery is due to plant responses and not to virus attenuation (Bennett et al., 1936). However, the results obtained in this study indicate that the recovery phenotype is enhanced by mutations in the L2 and L3 ORFs. This suggests that the L2 and L3 mutations may sufficiently attenuate the virus to allow plants to outgrow the infection. On the other hand, these proteins might be 'virulence factors' involved in maintaining the diseased state, and in the absence of these proteins the plant is able to rapidly recover from the disease condition. It is possible that the L2 and L3 proteins interact with and inhibit a plant defense pathway and so prolong infection.

The replication efficiency of the BCTV mutants was studied in protoplasts derived from *N. tabacum* suspension cells. The data indicate that the L2 protein can be dispensed with without affecting the accumulation of the various forms of BCTV DNA. There is no indication that L2 function is necessary for the expression of rightward genes. Unlike R1 and R2 mutants, L2 mutants show wild-type levels of replication in protoplasts (Briddon et al., 1989; Hormuzdi and Bisaro, 1993), and L2 mutants are infectious whereas R1 and R3 mutants are not. Also, western blot analysis of total protein preparations isolated from leaf discs revealed that L2-1 inoculated tissue contained wild-type levels of BCTV coat protein (data not shown).
<table>
<thead>
<tr>
<th>Protein</th>
<th>Residue</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>SqLCV</td>
<td>1</td>
<td>KVP-----S1K AGHRIAKRA VRRRIDLDC</td>
</tr>
<tr>
<td>TYLCV</td>
<td>1</td>
<td>TSHCSQV1K VQHKIAKKK KRRRVIDLDC</td>
</tr>
<tr>
<td>TGMV</td>
<td>1</td>
<td>TPF-----S1K AGHRAAAKRA IRRRIDLNC</td>
</tr>
<tr>
<td>ACMV</td>
<td>1</td>
<td>QHSTQVPIK VHRQVKIER IRRRVDVC</td>
</tr>
<tr>
<td>BGMV</td>
<td>1</td>
<td>QPF-----S1K AGHRIAKHA IRRRIDLNC</td>
</tr>
<tr>
<td>CONSENSUS</td>
<td>5</td>
<td>--P------S1K AGHRIAKRA IRRRIDL-C</td>
</tr>
</tbody>
</table>

** BCTV-CAL          | 1       | MENHVLKVV SKVRSRITIQQ HRFKVTIQP |
** BCTV-CFH         | 1       | MENHVLKVV SPVYVAYIQQA RLRTSSS  |
** BCTV-LOGAN       | 1       | MENHVLKVV SPVYVAYIQQA RLRTSSS  |

** FIGURE 36. Comparison of BCTV L2 protein with AL2 protein sequences. The consensus sequence is derived from a comparison of the AL2 proteins located on the genomes of SqLCV, TYLCV (Israeli isolate), TGMV, ACMV, and BGMV. Also shown are the sequences of the California (CAL), CFH, and Logan strains of BCTV. Asterisks are located above the positions of cysteine and histidine residues which may form a zinc finger DNA binding motif (Marcos D. Hartitz, personal communication). For the L2-3, L2-1, and L2-2 mutants, the L2 protein sequence is mutated from residues 14, 72, and 74, respectively. These residues are underlined in the Logan genome sequence. The arrows indicate the positions of the stop codon for L1 (leftward oriented arrow), and the initiation codon for L3 (rightward oriented arrow).
FIGURE 37. Comparison of the BCTV L3 protein with AL3 protein sequences. The consensus sequence is derived from a comparison of the AL3 proteins located on the genomes of ACMV, BGMV, SqLCV, TGMV, and TYLCV (Israeli isolate). Also shown is the L3 protein sequence of the California (CAL), CFH, and Logan strains of BCTV.
Despite these observations, the possibility that L2 protein may have some small quantitative effect on rightward gene expression cannot be ruled out. However, these results are consistent with earlier observations of Stanley et al. (1992a), and suggest that if transactivation of the R1 gene is needed for adequate synthesis of coat protein, then a protein other than L2 is involved. A likely candidate for such a transactivator may be the L1 gene product which, in subgroup I geminiviruses, is required for expression of the coat protein gene (Hofer et al., 1992).

Results obtained from protoplast analysis indicate that the L3 protein is required for efficient replication of the genome. In keeping with this conclusion, L2/3-1, L2/3-2, and L3 mutants showed about a three fold to five fold reduction in total DNA levels. Thus the L3 protein of BCTV and the AL3 protein of the bipartite geminiviruses, which have very similar amino acid sequences (Fig. 37), have a similar phenotype in protoplasts and probably are involved in the same pathway and possess similar biochemical properties. That L3 protein can complement a TGMV AL3 mutant, and vice versa, lends strong support to this view. In N. benthamiana plants, TGMV AL3 mutants show an attenuated phenotype. BCTV L3 mutants infect N. benthamiana plants with a virulence similar to wild-type virus early after inoculation, but the plants recover from the infection and are capable of further growth with little or no evidence of a BCTV infection. Thus, the overall phenotype of L3 mutants also is a reduction in symptom severity. Interestingly, symptom attenuation in the case of BCTV occurs only after the establishment of a wild-type infection. In this respect BCTV L3 mutants differ from TGMV AL3 mutants which are attenuated throughout the infection cycle. The reasons for this difference between the two geminivirus mutants is not known.

In summary, the analysis of the L2 and L3 ORFs indicates that neither is required for infectivity in N. benthamiana or sugarbeet plants. BCTV genomes
containing disruptions in both ORFs are as virulent as genomes disrupted in only one. The studies also indicate that the BCTV L3 and TGMV AL3 proteins are functionally equivalent.
CONCLUSION

The research described in the previous chapters utilized a mutagenesis approach to determine the functions of some of the TGMV and BCTV genes. These genes were categorized into determinants for the processes of replication and spread based upon the behavior of mutants in a transient protoplast replication assay and in *N. benthamiana* and sugarbeet plants. A brief summary of the major conclusions of these studies is given below.

The TGMV BR1 and BL1 genes, and BCTV R1 and R3 genes, are needed for movement of the virus in the host since mutations in these genes result in a loss of infectivity without disrupting replication of the virus. The BCTV R2 gene product is involved in maintaining a balance between ss- and dsDNA synthesis. Based upon the increase in dsDNA levels and the concomitant reduction in ssDNA levels associated with mutations in this gene, it is hypothesized that R2 protein regulates the switch between RF→RF synthesis to RF→SS synthesis. Interestingly, genomes bearing lesions in the R2 ORF are capable of eliciting an asymptomatic infection in *N. benthamiana* plants, but are incapable of systemic spread in sugarbeet plants. Thus, the requirement of R2 protein for replication/movement of the virus varies in its acuteness depending upon the host, and suggests that ssDNA may be involved in virus movement. It has also been determined that the BCTV L2 and L3 genes are not required for replication or spread. The enhanced recovery phenotype exhibited by *N. benthamiana* plants inoculated with L2 and L3 BCTV mutants is speculated to be due either to virus attenuation or to an active plant defense mechanism that is able to overcome the effects of virus infection in the absence of the L2 and L3 proteins.
Thus, the BCTV L2 and L3 gene products appear to be virulence factors. However, these experiments were unable to distinguish whether an L2 gene product plays a direct role in pathogenesis, or whether the lesions introduced in the L2 ORF affected expression of the L3 gene product in some subtle manner. Therefore, the results of these experiments must be interpreted with caution. Finally, it was determined that the BCTV L3 and TGMV AL3 proteins are functionally equivalent, implying that these proteins play similar roles in the replication cycles of their respective viruses.

Our current understanding of the replication cycles of TGMV and BCTV, emphasizing the putative roles of the various viral gene products, is shown in Figures 38 and 39. These Figures summarize the numerous studies on geminivirus gene function referenced in the preceding chapters. Upon entry into a plant cell, the virus particle is uncoated and the ssDNA genome is released. Thereafter the genome is replicated by a rolling circle mechanism utilizing the host replication machinery in conjunction with the AL1, AL2, AL3, and AR1 proteins for TGMV, and the L1, L3, R1, and R2 proteins for BCTV. As shown in Figure 38, the AL1 protein is responsible for regulating its own expression as well as that of AR1 protein (Sumter et al., 1993; F. Meyer and D. Bisaro, personal communication), and the AL2 protein is required for the expression of the AR1 and BR1 proteins. Based upon the extensive similarity between the amino acid sequences of the BCTV L1 and the TGMV AL1 proteins, the BCTV L1 protein may likewise regulate cellular levels of L1 and R1 proteins (Fig. 39), although this regulatory activity has yet to be demonstrated. While the overall replication scheme is similar, a significant difference between the two viruses concerns the regulation of ssDNA accumulation later in cycle. In TGMV, AL2 protein is needed for the expression of the coat protein (AR1) and BR1 protein, and the presence of AL2 protein may delineate the early and late phases of replication (Fig. 38). On the other hand, in the case of BCTV, the R2
FIGURE 38. Replication cycle of TGMV. This figure summarizes the numerous genetic and biochemical studies on TGMV. The putative roles of the viral gene products in the replication cycle of TGMV are indicated. Refer to the text for a description of key features of the replication cycle.
BCTV Replication Cycle

Virion → Inoculation
          ↘
          Uncoating → Acquisition
            ↖
          Encapsidation

Rolling Circle Replication

Host Proteins
pl1, pl3

ssDNA

Host Proteins
pl1, pl3

(ds RF)

(pl1)

(pl3)

(pl2, pl4)

(+)

(-)

Transcription & Translation

Late (rep regulation)

pR1 (coat protein, movement)

pR2 (rep regulation)

pR3 (movement)

FIGURE. 39. Replication cycle of BCTV. This Figure summarizes the numerous genetic and biochemical studies on BCTV. The putative roles of BCTV gene products in its replication cycle are indicated. Refer to the text for a description of key features of the replication cycle.
protein may directly regulate the onset of ssDNA synthesis by blocking dsDNA synthesis (Fig. 39). Thus, the two viruses appear to have adopted two different strategies for regulating the switch from RF→RF synthesis to RF→SS synthesis. Consistent with this conclusion also is the fact that, despite the uncertainty regarding the role of BCTV L2 protein in virulence, there is no evidence that it is needed for coat protein synthesis. Figures 38 and 39 also illustrate the findings that the TGMV AL3 and BCTV L3 proteins enhance replication levels and are functionally equivalent; a notion that is directly tested in Chapter IV. And finally, the Figures also illustrate that the TGMV BR1 and BL1 gene products, and the BCTV R1 and R3 gene products are involved in movement.

The studies described in the preceding chapters have further defined the roles of the concerned TGMV and BCTV genes and, in doing so, have contributed significantly to our present knowledge of the replication cycles of the subgroup II and III geminiviruses. This knowledge now provides a conceptual framework for further genetic and biochemical investigations of viral gene expression and DNA replication, and cell biological investigations of systemic spread. In addition, future work will focus on the interactions of viral proteins and host cell proteins leading to productive infection and the development of disease. A full understanding of these processes will require the identification and characterization of participating plant processes and components. In this regard, the identification of a single locus conferring resistance to BCTV in the genetically tractable plant Arabidopsis thaliana may represent an important advance (Lee et al., 1993).
REFERENCES


tumefaciens containing either monomeric or dimeric viral DNA. Plant Mol. Biol. 11, 795-803.


