CHARACTERIZATION OF THE RNA-DEPENDENT RNA POLYMERASE GENE OF CITRUS TRISTEZA CLOSTEROVIRUS

By

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To my wife Mehtap

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Tristeza disease is the most destructive and economically important problem in most citrus producing regions of the world. The disease is caused by citrus tristeza closterovirus (CTV) which is a single-stranded positive-sense RNA virus classified in the genus *Closterovirus*. The development of efficient control measures for CTV requires the characterization of genes encoded by the viral genome and a better understanding of how the virus replicates and causes disease. To improve the molecular understanding of CTV, the RNA-dependent RNA polymerase (RdRp) gene, which is required for the replication of the virus, was characterized.

To study the sequence variability in the RdRp gene of biologically and geographically different isolates of CTV, the RdRp genes from ten different strains were amplified by reverse transcriptase- polymerase chain reaction (RT-PCR) and sequenced. Analysis of the nucleotide and amino acid sequences showed that the RdRp gene was highly

conserved among all isolates of CTV. Evolutionary relationships among different isolates was predicted using phylogenetic analysis of the RdRp sequences. The RdRp gene from the Florida quick decline (QD) isolate T36 of CTV, was cloned and expressed in *E. coli* and polyclonal antiserum specific to the expressed RdRp fusion protein was produced. Using this antiserum, the RdRp was detected in CTV-infected tissue and found to be localized mostly in the membrane fraction of infected citrus cells.

It has been proposed that the RdRp gene of CTV is expressed by a +1translational frameshift, however, the occurrence of the +1 frameshift has not been demonstrated for CTV or any other closterovirus. The +1 translational frameshift was demonstrated by an *in vitro* transcription and translation method. An *in vivo Agrobacterium*-mediated transient expression assay was developed to investigate the involvement of the CTV sequences in the +1 frameshift. Deletion studies in the transient assay showed that 123-nucleotide overlapping sequence of the ORFs 1a and 1b was necessary and sufficient for the +1 frameshift. It was found that the 123 nt region contains a conserved stem loop structure, and that this conserved stem loop structure is required for the +1 frameshift.

To explore the possibilities of replicase- mediated pathogen-derived resistance against CTV, five different constructs of the RdRp gene of CTV, including full length, untranslatable, antisense and two modified RdRp genes (with mutations and a deletion at the GDD motif) were produced. Following transformation of grapefruit (*Citrus Paradisi* cv. Duncan) seedling segments using *Agrobacterium* containing these constructs, a number of transgenic plants were regenerated and established in the greenhouse. The transgenic nature of most of these plants were confirmed by PCR amplification of the GUS and RdRp genes from their genomic DNA.

CHAPTER 1
INTRODUCTION

Citrus is one of the most widely grown and economically important fruit crops in the world with an annual production of about 100 million metric tons. The world citrus production has been increasing steadily to accommodate growing demands of domestic and international markets. Citrus is not only economically important for generation of income and foreign trade but also very important in terms of nutrition. The United States (US) is the second largest citrus producer in the world, and the annual retail value of citrus grown in the US alone is about $20 billion. Thus citrus production makes a significant contribution to the economy of citrus growing states, especially the state of Florida where the majority of the US citrus is produced.

Tristeza disease of citrus, caused by citrus tristeza virus (CTV), is the most destructive and economically important disease in most citrus producing regions of the world. In the past, destructive epidemics of tristeza disease severely damaged or destroyed the citrus industries on sour orange rootstock by killing millions of citrus trees grafted in Brazil, Argentina, Venezuela, and Spain. In fact, the name tristeza, which means sadness in Spanish and Portugese, was given after the destructive epidemics in South America. The disease is now found in almost all citrus growing regions including Florida. It can significantly reduce yield and fruit quality and has become a limiting factor for commercial citrus production in some regions. The importance and destructiveness of tristeza disease

may increase and reach a new level in Florida due to the recent invasion of the brown citrus aphid (*Toxoptera citricida*), the most efficient vector of CTV.

Even though several strategies have been developed for managing the tristeza disease, there is no effective measure for controlling or eliminating CTV from citrus orchards. The currently available management strategies of quarantine and eradication are only effective for regions where CTV is not present or it has been just introduced. Tolerant rootstocks and mild strain cross protection are used in some regions where CTV is endemic; however, these strategies are not effective against all strains of CTV. As in other diseases, genetic resistance is the most effective strategy for controlling tristeza disease of citrus. Although a single dominant gene conferring resistance to CTV is found in *Poncirus trifoilata*, which is a close relative of citrus, integration of this gene into commercially important citrus scions has been hindered by the problems associated with conventional citrus breeding. The resistance gene has been mapped using molecular markers, but the isolation, characterization and use of this gene for developing CTV resistant citrus plants will still require many years of research. In recent years, advances in molecular biology, plant transformation and tissue culture techniques have provided new and more efficient approaches, such as genetic engineering, to overcome the limitations of conventional breeding. Using genetic engineering, transgenic plants with a defined trait can be developed by incorporating a specific gene from a different source into the plant genome without altering the other desirable characteristics. Genetically engineered resistance has been developed by transforming plants with genes or sequences from pathogens, which is termed pathogen-derived resistance (PDR). Different crop plants resistant to many viruses have already been successfully developed by transforming with viral genes. Hence, the genetic

transformation of citrus with sequences from the CTV genome has great potential for developing PDR against CTV.

As with other viruses, development of efficient control measures for CTV requires the characterization of the genome and a better understanding of the biology of the virus. Citrus tristeza virus is a positive-stranded RNA virus in the genus *Closterovius*. It is the largest known plant RNA virus with about 20 kb genome. Besides its large genome size, the genome organization, number and functions of the genes encoded by the genome, mechanisms of gene expression, and population complexity and unusual sequence variation among isolates are all important aspects of CTV biology. Genetic analysis of the CTV genome and understanding of the mechanisms of replication and pathogenesis of CTV were limited by the large genome size, low titer in virus-infected tissue, restriction to phloem tissue and the lack of a herbaceous experimental host. The genetic analysis of the CTV genome has recently begun with the development of a full length infectious cDNA clone and a protoplast system for replication of CTV.

In infected plants, CTV isolates consist of unusually complex populations of distinct genotypes. The complexity of CTV populations creates problems for diagnosis and strain identification, and also hinders the understanding of the mechanisms of pathogenesis. Sequencing and molecular characterization of genomes and/or individual genes of different isolates has improved our understanding of CTV biology, and this information should provide new approaches to control the tristeza diseases caused by CTV. The RdRp genes are highly conserved among all RNA viruses, and they are required for replication of these viruses. In addition, it has been demonstrated that the functional and modified sequences of the RdRp gene of many viruses confer resistance to virus infection in transgenic plants when

they are transformed with these genes. These general characteristics of the RdRp genes, in general, as well as the translational regulation of their expression, which is controlled by a +1 translational frameshift in closteroviruses, emphasize the significance of the RdRp gene for CTV and its potential use in development of resistance to the tristeza disease.

The overall objective of this study is to characterize the RdRp gene and its expression by the +1 translational frameshift, and to explore the possibilities of replicase-mediated resistance to CTV. The specific objectives are the following:

1) To determine the sequences of the RdRp genes from geographically and biologically different isolates and to study the sequence variation and relationships among different isolates of CTV.

2) To clone and express the RdRp gene of CTV in *Escherichia coli*, and to produce polyclonal antiserum against the expressed protein for the detection of the RdRp in CTV-infected plants.

3) To demonstrate the occurrence of the +1 translational frameshift proposed to be required for the expression of the RdRp gene of CTV and to characterize the sequences involved in the +1 translational frameshift.

4) To transform Duncan grapefruit plants with different constructs of the RdRp gene from CTV and to regenerate and produce transgenic plants potentially resistant to CTV.

CHAPTER 2
REVIEW OF LITERATURE

Tristeza Disease

Tristeza is one of the most destructive and economically important diseases of commercial citrus worldwide (Bar-Joseph et al. 1989). Although the disease is believed to have originated in southeast Asia (Wallace et al. 1956), it was first reported as a decline of citrus scions grafted on sour orange rootstock in South Africa around 1910 (Weber, 1943). A similar decline disease in the 1930s causing epidemic losses of sweet orange trees on sour orange rootstock was reported in Argentina and Brazil. Tristeza was first thought to be a rootstock-scion incompatibility, or a nutritional problem or a root disease. In 1946, Meneghini transmitted the disease by aphids and experimentally proved it to be of viral origin (Bar-Joseph et al. 1989; Lee and Rocha-Pena, 1992). Earlier reports of incompatibility, nutritional problems and quick decline are now all recognized as different forms of the same viral disease caused by citrus tristeza virus (CTV) (Grant et al. 1951; McClean, 1950). Some other disorders found later in citrus, such as stem pitting (Da Graca et al. 1984) and seedling yellows (Roistacher, 1982), also are associated now with CTV.

In the United States, quick decline of citrus on sour orange rootstock was first reported in California (Fawcett and Wallace, 1946) and later in Florida (Grant, 1952). It was suggested that the disease was introduced into California with Meyer lemon introductions from China in 1908, and then introduced into Texas and Florida by the movement of Meyer lemon (Wallace and Drake, 1955). Tristeza is now present in most

world, with the exception of some Mediterranean and Central American countries, and some isolated islands (Lee and Rocha-Pena, 1992).

Citrus Tristeza Virus

Taxonomy

Based on distinct morphological, biological and molecular characteristics and phylogenetic analysis, CTV is classified in the genus *Closterovirus* in the *Closteroviridae* family of the positive-stranded plant RNA viruses (Bar-Joseph et al. 1979; Koonin and Dolja.,1993; Dolja et al. 1994). Citrus tristeza virus and the other members of the closterovirus group are characterized by long flexuous rod-shaped particles, a large undivided genome, association with phloem tissue of their hosts, semi-persistent mode of transmission and vector specificity, and typical inclusion bodies produced in the infected cells (Bar-Joseph et al. 1979).

Morphological Characteristics of CTV

Citrus tristeza virus has long thread-like, flexuous, filamentous particles about 2000 nm by 11 nm (Bar-Joseph et al. 1979). Virions of CTV consist of one single-stranded positive-sense RNA molecule encapsidated with two capsid proteins (CPs). The 25 kDa major CP encapsidates about 95% of the genome, and the remaining portion of the genome is encapsidated by the 27 kDa minor CP on one end of the virion (Febres et al. 1996). The virion has helical symmetry with a primary pitch of 3.5 to 3.7 nm and 8.5 to10 CP subunits per helical turn, respectively (Bar-Joseph., 1972). Long flexuous particles of CTV can easily be observed with an electron microscope in leaf-dip and partial preparations from the phloem tissue of infected citrus plants (Bar-Joseph et al. 1972; Febres et al. 1996).

Biological Characteristics of CTV

Host range of CTV

Citrus tristeza virus has a narrow natural host range essentially limited to plants in a single genus, the genus *Citrus*, in the family Rutaceae. Citrus tristeza virus is able to infect most species, varieties and hybrids of *Citrus* (Muller and Garnsey, 1984) as well as some close relatives of citrus including *Aglopsis chevalieri*, *Afraaegle paniculata*, *Pamburus missiones* (Knorr, 1956) and *Aegle marmelos* (Muller and Garnsey, 1984). On the other hand, some citrus relatives, such as *Severinia buxifolia*, *Swinglea glutinosa*, *Poncirus trifoliata*, and hybrids between *P. trifoliata* and sweet orange or grapefruit, are resistant or immune to CTV infection. The virus is not able to replicate or cause symptoms in these hosts (Garnsey et al. 1987a). To determine the host range and identify a herbaceous experimental host, CTV has been inoculated to about 200 plant species outside of the family Rutaceae, but the virus multiplied only in some species of *Passiflora*, particularly in *Passiflora gracilis* (Muller et al. 1974).

Cytopathic effects of CTV

Plant viruses usually produce inclusion bodies which are distinctive intracellular structures of aggregates of virus particles and related proteins found in the virus-infected cells. Citrus tristeza virus produces two types of inclusion bodies characteristic of members of the closterovirus group. Inclusion bodies appear as aggregated virus particles in cross-banded arrays in the phloem and associated cells of the CTV-infected plants. They may be seen by Azure A staining, *in situ* immunofluorescence, and light and electron microscopy (Garnsey et al. 1980; Brlansky, 1987; Brlansky et al. 1988). Recently, it was demonstrated that a 20 kDa protein, encoded by ORF10 of CTV, is the major component of the characteristic amorphous inclusion bodies present in CTV infected cells (Gowda et al. 2000). Studies with

different isolates of CTV indicated a positive relationship with the number of inclusion bodies to increasing strain severity and virus titer in different host plants. Thus detection of CTV inclusions has been used as a rapid method for diagnosis and strain differentiation (Brlansky and Lee, 1990). A second type of inclusion occurring in CTV-infected plants consists of groups of fibril-containing vesicles that are surrounded by cytoplasmic membranes (Chen et al. 1971). Similar vesicles are also produced in plants infected with other closteroviruses and presumably contain double-stranded RNA ; thus, they might be involved in virus replication (Coffin and Coutts, 1993).

Symptoms of CTV

A variety of symptoms is produced in different hosts infected with different CTV isolates. Symptoms caused by different isolates of CTV are divided into five major groups including mild vein clearing, seedling yellows (SY), quick decline (QD), stem pitting on grapefruit (SP-G) and on sweet orange (SP-O). Mild isolates of CTV express weak vein clearing and flecking on leaves of Mexican lime and produce no noticeable symptoms on commercial citrus. The SY is expressed as severe chlorosis and dwarfing of sour orange, lemon or grapefruit. The SY symptoms are easily observed under greenhouse conditions, but they are not usually seen in the field (Roistacher, 1982). The QD is more severe and can occur on sweet orange, mandarin and grapefruit scions grafted on sour orange rootstock. The QD is caused by a virus-induced phloem necrosis in the bark of the rootstock just below the graft union that prevents the movement of carbohydrates from the canopy to the root system. Lack of carbohydrates in the root system causes the roots to degenerate and inhibits formation of new fibrous roots that result in the decline of trees (Garnsey et al. 1987). The stem pitting symptoms are characterized as stunting, chlorosis, reduced fruit number and size, and typical

pitting on stems, major branches and twigs of most citrus scions, especially grapefruit and sweet orange, regardless of their rootstock. The pits are caused by the collapse of the phloem tissue and can be observed easily when the bark of twigs is peeled (Da Graca et al. 1984). A standardized set of indicator plants has been established to determine the biological characteristics of CTV isolates. The indicator plants include sour orange for SY, sweet orange grafted on sour orange for QD, Duncan grapefruit for SP-G, Madam Vinous for SP-O, and Mexican lime as an universal indicator (Garnsey et al. 1987).

Transmission of CTV

Citrus tristeza virus is readily graft transmitted among the compatible *Citrus* spp. using phloem-containing tissue (Roistacher, 1976). The virus was mechanically transmitted to several hosts by stem-slash inoculation with inoculum preparations of different levels of purity (Muller and Garnsey 1984). In nature, CTV is transmitted by aphid vectors including *Toxoptera citricida*, *Aphis gossypii*, *A. spiraecola*, and *T. aurantii* (Roistacher and Bar-Joseph, 1987; Brunt et al. 1990).

Citrus tristeza virus is transmitted in a semi-persistent manner by its aphid vectors in nature. Efficient transmission of CTV requires 30 min to 24 hrs of acquisition feeding (Roistacher and Bar-Joseph, 1987), and the aphid retain the ability to transmit for one to three days (Yokomi and Garnsey, 1994). *Toxoptera citricida*, commonly called the brown citrus aphid (BCA) was first recognized in 1946 in Brazil, and is the most efficient vector of CTV (Lee and Rocha-Pena, 1992; Bar-Joseph, 1989). Comparative analysis of transmission efficiency with different aphid species showed that *T. citricida* transmits CTV with about 16 to 20% efficiency using a single aphid transmission, compared to 0.5 to 1.4% efficiency for *Aphis gossypii* (Yokomi et al. 1994). Transmissibility of CTV by its aphid vectors,

particularly *A. gossypii*, is affected by the strain of the virus (Roistacher and Bar-Joseph, 1984), donor and receptor host species and environmental conditions, such as temperature (Bar-Joseph et al. 1977). Over the years, the BCA has been responsible for natural spread of CTV in most citrus growing areas including South America (Costa and Grant, 1951), South Africa (McClean, 1975), Australia, and Asia (Tanaka, 1969). In the 1990s, the BCA moved northward from South America invading Central America and the Caribbean Basin (Yokomi et al. 1994; Rocha-Pena et al. 1995) and was reported in Florida in 1995 (Fasulo and Halbert,. 2000).

Aphis gossypii is the principal vector of CTV in California (Roistacher and Bar-Joseph, 1984; Roistacher and Bar-Joseph, 1987), Spain (De Mendoza et al. 1984), and Israel (Raccha et al. 1976) where the BCA is not present. Although *A. spiraecola* and *T. aurantii* are found in Florida (Norman and Grant, 1959), California (Dickson et al. 1951), Israel (Raccha et al. 1976) and Spain (De Mendoza et al. 1984), they are less efficient vectors and have little significant effect on the spread of CTV (Roistacher and Bar-Joseph, 1987).

Biochemical Properties of CTV

Citrus tristeza virus is the largest known plant virus, containing about 20 kb of single-stranded positive-sense RNA genome (Karasev et al. 1995). It has been proposed that the unusually large genome of CTV and other closterovirus have evolved from ancestral tobamo-like viruses by the shuffling of genome elements, gene duplication and divergence, acquisition of novel genes from host cells and other viruses and the development of new gene expression strategies (Dolja et al. 1994). Besides its large genome size, the genome organization, number and functions of the genes encoded by the genome, mechanisms of gene expression, and population complexity and unusual sequence variation among isolates are all important

molecular and biochemical aspects of CTV. Their role in the tristeza disease is only now being investigated and determined (Satyanarayana et al. 1999).

Genome Organization of CTV

The complete genome sequence of four CTV isolates, each with a different biological activity, were determined (Karasev et al. 1995; Mawassi et al,1996; Yang et al. 1999; Vives et al, 1999). Based on sequence analysis, the genome of CTV is organized into 12 open reading frames (ORF) potentially encoding 17 protein products, plus 3' and 5' untranslated regions (UTR). The first ORF (ORF1a) encodes a 369-kDa putative polyprotein containing two papain-like proteases, methyltransferase and helicase domains. The ORF1b codes for the putative RNA-dependent RNA polymerase (RdRp) which is thought to be expressed by a +1 frameshift at the 3' terminus of ORF1a. (Karasev et al. 1995). The remaining ten ORFs are individually expressed through sub-genomic mRNAs and encode 33, 6, 65, 61, 27, 25, 18, 13, 20 and 23 kDa protein products from the 5' to 3' direction (Pappu et al. 1994; Karasev et al. 1995).

The genome of CTV can be divided into four modules which are conserved among the closteroviruses: the core module, the upstream accessory module, the chaperon module and the CP module. The core module consists of the domains of methyltransferase, helicase and RdRp, which are all associated with virus replication and conserved throughout the alphavirus supergroup of the RNA viruses. The upstream accessory module includes two leader papain-like protease domains probably involved in polyprotein processing. The chaperone module includes a small protein with membrane-binding domains, one heat shock protein 70 homolog (HSP70) and one protein distantly related to heat shock protein HSP90. The CP module consists of the major and the minor CP genes and four additional 3' terminal ORFs.

Characteristics and Functions of Genes of CTV

The CTV polyprotein is about 369 kDa and contains two putative proteases, a methyltransferase and a helicase domain. The protease domains are similar to papain-like thiol proteases with a cysteine and histidine residue at the catalytic site which are probably involved in processing of the polyprotein of CTV. Significant similarities in their size and amino acid sequence suggests that the two proteases of CTV may have evolved by gene duplication (Karasev et al. 1995). The methyltransferase and helicase domains are generally required for virus replication and are associated with the RdRp. The methyltransferase and helicase of beet yellows virus (BYV) were detected in infected tissue, and both proteins were found to be associated with membrane compartments of the infected cells. Detection of these proteins in the infected tissue demonstrates that they are processed from the polyprotein by a host or virus encoded protease (Erokhinia et al. 2000).

The putative RdRp of CTV is encoded by ORF1b and is proposed to be expressed by a +1translational ribosomal frameshift at the 3' terminus of ORF1a (Karasev et al. 1995). Deletion analysis of the genome of the infectious CTV clone demonstrated that only the gene products of the ORF1a and 1b, which includes protease, methyltransferase, helicase and RdRp, are required for replication of CTV in *Nicotiana benthamiana* protoplasts (Satyanarayana et al. 1999). The p33 gene is not found in other members of the closterovirus group and has no homology with any known sequences. The p6 gene encodes a small hydrophobic protein containing sequences similar to membrane-spanning domains and may be associated with membranes (Pappu et al. 1994). The p65 gene contains a conserved ATPase domain and shows high homology with the cellular heat shock protein HSP70. The p61 gene of CTV and its analogs in some, but not all, closteroviruses has conserved sequences with

limited homology to another cellular chaperone HSP90 (Pappu et al. 1994). The HSP70s are ubiquitous molecular chaperone-like proteins and participate in a wide range of cellular processes including protein folding, assembly, translocation and intercellular transport (Feder and Hofmann, 1999). Recent studies revealed the role and importance of the HSP homolog proteins in CTV and other closteroviruses. The analog of CTV p65 gene in BYV was able to complement the movement proteins of other viruses, and it is involved in cell-to-cell movement of BYV (Agranovsky, 1999). In addition, analogs of CTV p65 and p61 proteins were found to be physically associated with virion particles in other closteroviruses (Tian et al. 1999; Napuli et al. 2000). Furthermore, it was demonstrated for CTV that the p65 and the p61 proteins are required for assembly of the CTV virions. Deletion of these genes significantly reduced the formation and passage of full-length CTV virions (Satyanarayana, 2000).

The 25 kDa protein encoded by the ORF7 is the major capsid protein (CP) (Sekiya et al. 1991) which encapsidates most of the CTV genome. The 27 kDa protein identified as minor capsid protein is a diverged copy of the major CP (Febres et al. 1994) and coats only 5% of the genome at one terminus (Febres et al. 1996). Identification of a conserved epitope in most CPs of severe isolates of CTV, but not in mild isolates, indicated that CTV may have pathogenicity determinants associated with the major CP gene (Pappu et al. 1993). The 20 kDa protein shows high affinity for itself and accumulates in the characteristic amorphous inclusion bodies present in CTV infected phloem cells. Thus, this gene is involved in the formation of inclusion bodies in the infected cells (Gowda et al. 2000). The 23 kDa protein, encoded by the 3' terminal gene, is localized predominantly in the cytoplasm of CTV-infected cells (Pappu et al. 1997). Recently the recombinant 23 kDa protein was demonstrated to be

able to bind both single and double-stranded RNA in a non-sequence specific manner, suggesting that the 23 kDa protein of CTV is an RNA-binding protein. The RNA-binding activity was mapped to a region containing a cluster of positively-charged amino acids and sequences similar to putative zinc-finger domains (Lopez et al. 2000). The rest of the genome has no homology with any known sequences in the databases (Pappu et al. 1994; Karasev et al. 1995).

Replication of CTV

Replication of the genome is the fundamental aspect in the biology of all viruses. The replication of positive-stranded RNA viruses takes place in two main stages. First, the negative or complementary-strand RNA is synthesized from the genomic positive-strand RNA template, and then the negative-strand of RNA is used as a template to produce positive-strand RNA progeny. Studies of replication of a number of animal and plant RNA viruses showed that virus replication requires several viral encoded proteins including RdRp, helicase and MT, cis-acting viral sequences, such as the 3' and 5' terminal sequences and internal sequences, as well as host proteins (Buck, 1996).

The large genome size, low titer and restriction to phloem tissue, and the lack of an herbaceous experimental host have greatly hindred genetic analysis and understanding of the replication of CTV. Recent development of a full-length infectious cDNA clone of CTV (Satyanarayana et al.1999) and protoplast systems for CTV replication (Price et al. 1996; Navas-Castillo et al. 1997) have facilitated the genetic analysis of the CTV genome. Deletion analysis of the infectious cDNA clone of CTV demonstrated that only ORF1a and 1b, coding for the replication-associated proteins, as well as the 3' and 5' UTRs of the genome were essential for replication of CTV in *N. benthamiana* protoplasts. A smaller CTV replicon

with a 10 kb genome, which contains only the ORF1a\b and the 3' and 5' UTRs generated by deletion of ten 3' ORFs replicated more efficiently than the full-genomic RNA in tobacco protoplasts (Satyanarayana et al. 1999). Analysis of the 3' and 5' UTR sequences from different isolates showed that the 3' UTR is highly conserved, whereas the 5' UTR sequences were highly variable among different isolates of CTV. However, the presence of a conserved secondary structure was predicted in the 5' UTR of all CTV isolates. This structure consists of two stem loops, and it may function as a cis-acting element during replication (Lopez et al. 1998). To determine the effect of UTRs for replication, the UTRs of the CTV replicon were substituted with the 3' and 5' UTRs from different isolates. Different 3' UTRs still enabled replication, but the replication efficiency of the replicon with heterologous 5' UTRs was significantly reduced. The reduction was proportional to the extent of sequence variability among the 5' UTRs indicating that, in addition to the secondary structure, the primary structure of the 5' UTR is also important for CTV replication (Satyanarayana et al. 1999). During CTV replication, different forms of double-stranded RNAs and a number of subgenomic (sg) and defective RNAs are produced in addition to the full-length replicative genomic RNA.

Defective RNAs Associated with CTV

Defective RNAs (D-RNAs) are non-autonomous RNA molecules derived mainly or entirely from genomic nucleotide sequences of the virus and are associated with certain viruses. The D-RNAs have been found to be associated with a number of animal viruses (Huang, 1993), and some positive-stranded RNA plant virus groups, such as tombusviruses, carmoviruses and potexviruses (Simon and Bujarski, 1994). Recently, a number of defective D-RNAs ranging from 1.5 to10 kb were detected in CTV infected plants. These D-RNAs show significant sequence homology with different portions of both the 3' and 5' ends of the

CTV genome, indicating that D-RNAs were formed by extensive internal deletion from the genomic RNA of CTV (Mawassi et al. 1995; Yang et al. 1997; Ayllon et al. 1999; Yang et al. 1999). Two different mechanisms of recombination were proposed for the generation of D-RNAs detected in CTV-infected samples. It was found that a substantial number of D-RNAs had variable 5' ends, but a common 3' terminus containing the complete sequence of the sgRNA for the ORF11. Based on this observation and the presence of an extra cytosine nucleotide at the junction site, it was proposed that these D-RNAs were generated by "minus strand jumping" during virus replication (Yang et al. 1997). Finding of the tetra-nucleotide AAGC direct repeat located in the flanking or in the vicinity of the junction sites of some D-RNAs suggested that these D-RNAs were generated by replication-driven template switching during virus replication (Ayllon et al. 1999). Since D-RNAs lack the genes necessary for replication, they are non-autonomous and require a helper virus for their replication in the cells. Homologous and heterologous D-RNAs of different CTV isolates were replicated *in trans* by the helper virus in the naturally infected plants and in tobacco protoplasts (Mawassi et al. 2000). The presence of D-RNAs in the virus-infected plant may interfere with virus replication and increase or decrease symptom severity. It was suggested that D-RNAs of 4.5 and 5.1 kb were associated with reduction of SY symptoms in some CTV isolates (Yang et al. 1999). However, there is no conclusive data for the involvement of D-RNA in symptom modulation in the CTV infected plants.

CTV Gene Expression Strategies

Based on phylogenetic analysis of sequences of the RdRp and other replication-associated proteins, the CTV strategy is similar to the alpha-like supergroup of positive-stranded RNA viruses (Koonin and Dolja, 1993). On the other hand, the organization and expression of the CTV genome are more similar to the coronaviruses. Both coronaviruses and

CTV use several different gene expression strategies including polyprotein processing, translational frameshifting, and subgenomic RNAs for expression of their large genomes.

Polyprotein processing

The ORF1a is expressed as a 349 kDa polyprotein containing two putative proteases, a methyltransferase and a helicase domain. In addition, the RdRp of CTV encoded by ORF1b is thought to be expressed by a +1translational ribosomal frameshift near the 3' terminus of ORF1a resulting in a 400 kDa polyprotein (Karasev et al. 1995). Individual domains become active proteins when they are released from the polyprotein by protease cleavage. Based on sequence analysis, the CTV genome encodes two papain-like proteases presumably for processing of the polyprotein. Two glycine residues located at position 484-485 and 976-977 from the N terminus of the polyprotein are predicted cleavage sites for these proteases. Thus processing of the 349 kDa polyprotein would produce two leader proteins of 54 and 55 kDa and a 240 kDa protein containing the MT and helicase domains. Detection of the product of both the MT and helicase domains of BYV as individual proteins in the infected tissue indicates that the polyprotein of BYV, and possibly CTV, are further processed by either viral or host proteases (Erokhinia et al. 2000).

Subgenomic RNA

Some viruses produce a set of subgenomic RNAs for translation of internal ORFs in their genome. Several sgRNAs were detected in CTV-infected tissue. Characterization of the CTV sgRNAs by Northern blot hybridization with gene-specific cDNA probes revealed the presence of a series of nine 3' co-terminal sgRNAs responsible for translation of ORFs 2 to 10, located at the 3' half of the genome. The pattern of 3' co-terminal sgRNAs was not affected by different citrus hosts (Hilf et al. 1995) and an identical pattern of sgRNA also was reported

in *N. bentamiana* protoplasts infected with CTV (Navas-Castillo et al. 1997). The relative amounts of the different sgRNAs were variable, with the sgRNAs for the p20 and p23 ORFs being the most abundant in both infected citrus tissue and tobacco protoplasts (Hilf et al. 1995; Navas-Castillo et al. 1997). Analysis of the 5' terminus of the two most abundant sgRNAs demonstrated that the p20 and p23 specific sgRNA have 48 and 38 nucleotide long 5' UTRs, respectively. The sequence of the 5' UTRs was colinear with the downstream sequence of their respective ORFs in the genome, indicating that the structure of sgRNAs of CTV is similar to that of alpha-like viruses that produce sgRNAs with different 5' UTRs colinear to their corresponding genomic sequences. Thus, they are different from coronoviruses, which produce sgRNAs with identical 5' UTRs derived from the 5' end of their genomic RNAs (Karasev et al. 1997).

Translational frameshifting

Ribosomal frameshifting is a directed change in the translational reading frame that allows production of a single protein from two overlapping genes. The frameshift can occur in either the 5' (+1 frameshift) or the 3' (-1 frameshift) direction (Brierley, 1995). A number of viruses and mobile genetic elements use the ribosomal frameshifting mechanism to control expression of their replicase gene at the translational level. This mechanism enables controlled low-level synthesis of the polymerase, which is needed in only small quantities (Brault and Miller, 1992). The -1 frameshift was demonstrated for animal retro- and coronaviruses (Brierley, 1995), yeast double-stranded RNA viruses (Dinman and Wicker, 1994), as well as in the plant *luteovirus*, *sobemovirus*, *carlavirus*, *enamovirus* and *dianthovirus* groups (Maia et al. 1996). The +1 frameshifting has been described in a few

organisms including yeast transposon TY, the copia-like element of Drosophila (Farabaugh, 1997), and it has been proposed for plant closteroviruses (Agranovsky et al. 1994).

It has been proposed that the putative CTV RdRp gene is expressed via +1 frameshift in the 3' terminus of ORF1a (Karasev et al. 1995). Based on sequence analysis of CTV and BYV, the frameshift is predicted to take place in CTV at nucleotide 9405, located in the overlap region of ORF1a and 1b. Although the overlap region of ORF1a and 1b is relatively conserved, there are significant differences between the BYV and CTV sequences around the predicted frameshift site. A UGA stop codon, a GGGUUU slippery sequence and a stem-loop structure were found in the predicted frameshift site of BYV, and they are proposed to be involved in the +1 frameshift of BYV (Agranovsky et al. 1994). The absence of these elements in CTV sequences indicated that the mechanism of the +1 frameshift differs between BYV and CTV. It was suggested that the frameshift occurs at the rare arginine codon CGG of CTV, which aligns with the UAG stop codon in BYV and then serves as a stop codon to pause the ribosome during translation, thereby inducing the +1frameshift in CTV (Karasev et al. 1995).

Sequence Variation Among CTV Isolates

Citrus tristeza virus isolates consist of unusually complex populations of distinct genotypes possibly contributed by the perennial nature and vegetative propagation of host plants, multiple aphid transmissions and genetic properties of the virus such as recombination and formation of D-RNAs. The complexity of CTV populations in the infected plants presents problems for diagnosis and strain identification, and also for understanding the mechanisms of pathogenesis and symptom induction in different host plants.

The complete genomic sequences were published for four biologically and geographically different isolates of CTV. These include T36, a quick decline isolate from Florida (Karasev et al. 1995); VT, a stem pitting and seedling yellows isolate from Israel (Mawassi et al. 1996); SY568, a stem pitting and seedling yellows isolate from California (Yang et al.1999); and T385, a mild isolate from Spain (Vives et al. 1999). The genome organization of all isolates was identical, and their genome lengths were very similar, ranging from 19,226 to 19,296 nucleotides. However, these isolates showed varying degrees of and unusual distribution of sequence diversity in their genomes.

Comparative analysis of genomic sequences of the CTV isolates revealed an unusual asymmetric sequence similarity along the T36 genome. The 3' halves of the genomes were highly conserved with over 90% sequence identity, but the 5' halves of the genomes had as low as 70% identity (Mawassi et al. 1996; Dodds et al.1997; Vives et al. 1999). The genomes of VT and T385 showed about 90% sequence identity evenly distributed throughout the genome. The genome of SY568 showed about 90% sequence identity toward both the 3' and 5' end of T385 genome, but the central parts of two genomes were more than 99% identical. From this data, it was inferred that the central portion of the SY568 genome results from RNA recombination between two CTV genomes (Vives et al. 1999). Analysis of the 3' and 5' UTR sequences from the genomic and partial sequences showed that the 3' UTR is highly conserved with above 97% identity among different isolates of CTV, but the 5' UTRs were highly variable, with as low as 42% sequence identity (Lopez et al. 1998;Vives et al. 1999). Using the variable 5' UTR sequences, CTV isolates were classified into three different groups containing mild isolates in one group (III) and severe isolates in the other two groups (I and II) (Lopez et al. 1998). Based on comparisons of representative genomic sequences

from the 5' and 3' regions of CTV isolates, formation of two groups of CTV isolates represented by VT and T36 was proposed. The proposed VT group contained isolates having relatively constant and evenly distributed genomic sequence divergence, and the T36 group consists of isolates having highly diverged 5' genomic sequences (Hilf et al. 1999).

Sequence variability among CTV isolates has been studied extensively using CP gene sequences. Comparison of the CP sequences from a number of biologically and geographically different isolates showed that the CP gene was highly conserved. Phylogenetic analysis of CP sequences generated distinct groups of CTV isolates with similar biological activities such as mild, quick decline and stem-pitting isolates (Pappu et al. 1993; Cevik et al. 1995). In addition, minor sequence differences were found in CP genes from different CTV isolates. Since these minor differences were conserved in a group of isolates with a specific biological activity, they may be associated with those biological characteristics of the CTV isolates (Cevik et al. 1996). Beside sequencing, several other methods including reverse transcriptase-polymerase chain reaction (RT-PCR) (Cevik et al. 1995), restriction fragment length polymorphism (RFLP) (Gillings et al. 1993), single-stranded conformation polymorphism (SSCP) (Febres, 1995; Rubio et al 1996), and hybridization with group or strain-specific probes (SSP) (Cevik et al. 1996) have been used to study the sequence variation in the CP and several other 3' genes of different CTV isolates.

Control of CTV

Several strategies have been developed for managing the tristeza disease. Different strategies are available for use based on the absence or presence of CTV in different citrus growing areas. Quarantine, budwood certification and/or clean stock programs are used to prevent introduction of CTV into countries where CTV is absent. In some regions where CTV

incidence is low, the disease can be managed by eradication or suppression programs, when combined with use of certification and clean stock programs. Use of CTV tolerant rootstocks, mild strain cross protection, and, in the future, genetically engineered resistance, combined with certification and clean stock programs are the potential control measures for those regions where CTV has become endemic (Lee and Rocha-Pena, 1992).

Mild Strain Cross Protection

Cross protection is a phenomenon in which a plant systemically infected with a mild strain of a virus is protected from subsequent infection or expression of symptoms of severe strains of the same virus, or closely related viruses (Fulton, 1986). Mild strain cross protection is the only available management strategy that can be implemented immediately to control CTV in Florida and other areas where such mild strains have been collected and evaluated. Cross protection has been used to control CTV on a large scale in commercial citrus plantations, particularly with Pera sweet orange in Brazil (Costa and Muller, 1980), grapefruit in Australia (Barkley et al. 1990), South Africa, Japan, *Citrus hystrix* in Reunion and limes in India (Lee and Rocha-Pena, 1992). Cross protection is now being applied in other citrus growing areas where CTV has become endemic, such as Florida (Lee et al. 1987; Lee and Brlansky, 1990). Mild strain cross protection is an effective control strategy for CTV in situations where the disease is endemic and impossible to control by eradication or suppression, and when cross protecting mild strains are available which are mild in all citrus cultivars in the region (Costa and Muller, 1980; Lee et al. 1987).

Use of CTV Tolerant Rootstocks

Most commercial citrus varieties and *Citrus* relatives are susceptible to CTV. However, some rootstocks are tolerant to CTV, meaning that the virus replicates in the host,

but no or minor disease symptoms are expressed in the infected plant. Some *Citrus* sp. generally used as rootstocks including *C. reticulata, C. volkameriana and C. jambhiri* (Rangpur lime) are naturally tolerant to QD-inducing isolates of CTV. Other hybrid rootstocks including citranges (*Citrus sinensis* X *Poncirus trifoliata*) and citrumelos (*C. paradisi* X *P. trifoliata*) have been developed and are used as CTV tolerant rootstocks to control CTV in some citrus growing regions. The presence of other economically important diseases, such as citrus blight, *Phytopthora sp.* and viroids, and undesirable horticultural characteristics limit the usefulness of these rootstocks in some citrus growing areas (Garnsey et al. 1987; Davies and Albrigo, 1994). In addition, some CTV isolates are able to induce the SP symptoms in the scions regardless of the tolerance of their rootstocks (Bar-Joseph and Lee, 1989). Thus, the CTV tolerant rootstocks do not give control against CTV-SP isolates in citrus growing areas where these isolates are widespread.

Breeding for CTV Resistance

There is no genetic resistance in the genus *Citrus* available that is effective against all CTV isolates. However, some citrus relatives, such as *Hesperethusa*, *Luvunga*, *Merope Oxantherea,*, *Severina*, *Swinglea* and *Poncirus*, are immune to CTV, meaning that the virus does not replicate in plants from these genera (Garnsey, 1987). All of these citrus relatives, except *Poncirus,* are sexually incompatible with *Citrus*. Thus, it is not possible to incorporate the CTV resistance into most commercially desirable citrus varieties by conventional breeding techniques. Genetic studies revealed that resistance found in *P. trifoliata*, which is sexually compatible with citrus, is controlled by a single dominant gene that prevents virus replication in the plant by an undetermined mechanism (Yoshida, 1996). *Poncirus trifoliata* has been crossed with different citrus species to introgress this CTV resistance into desirable citrus

cultivars. Although several CTV-tolerant rootstocks, such as citranges and citrumelos were developed as a result of these crosses, no scion variety with the CTV resistance and acceptable horticultural characteristics has been produced. The integration of the CTV resistance gene into commercially important citrus by classical breeding is difficult because of the problems associated with citrus breeding including large plant size, long juvenility period, polyembryony, heterozygosity, sterility, self- and cross-incompatibility and inbreeding depression (Soost and Roose, 1996). The CTV resistance gene in *P. trifoliata*, designated *Ctv*, has been mapped recently using molecular markers (Gmitter et al. 1996; Deng et al. 1997). Mapping the *Ctv* gene and identification of markers closely linked with the gene is useful for marker-assisted rapid selection of hybrids resistant to CTV and map-based cloning of the *Ctv* gene in the future. The cloned resistance gene can be introduced into the citrus genome by genetic transformation, which now offers a more efficient approach for development of CTV-resistant citrus cultivars.

Genetic Engineering for Virus Resistance

Recent developments in molecular biology and plant transformation techniques provide new approaches and open new possibilities for the generation and evaluation of sources of virus resistance outside of conventional breeding methods. Genetic engineering allows the development of transgenic plants with a defined trait by incorporating a specific gene into the plants genome without altering the other desirable characteristics. Virus resistance has been engineered by transforming plants with genes or sequences from viruses and/or other sources (Fuchs and Gonsalves., 1997). Virus resistance was engineered by expressing a number of genes from a variety of organisms including genes encoding anti-viral proteins such as ribosome inactivating proteins from pokeweed *Phytolacca americana*

(Lodge et al. 1993), rat 2'-5' oligoadenylate synthetase (Truve et al. 1993), human 2-5A synthetase and a 2-5A-dependent RNase L (Mitra et al. 1996; Ogawa et al. 1996), virus specific antibodies "plantibodies" (Voss et al. 1995), synthetic peptides (Marcos et al. 1995) defense related compounds (Herbers et al. 1996) and ribozymes (Nakamura et al. 1995). Although expression of these genes resulted in resistance to a specific virus or in some cases a diverse groups of plant viruses in the transgenic plants, the majority of transgenic crop plants engineered for virus resistance has been developed using sequences derived from plant viral genomes.

Pathogen-derived resistance

The pathogen-derived resistance (PDR) concept was first developed as a strategy to produce resistance against a specific or range of pathogens by transforming host cells with entire genes or sequences derived from the pathogen genome. It was proposed that expression of genes in an inappropriate amount, form, time, or location in the cell may interfere with the normal life cycle of the pathogen in their host and thereby induce resistance against the pathogen (Sanford and Johnson, 1985). Pathogen-derived resistance for a plant virus was first developed in 1986 (Powell-Abel et al. 1986) by demonstrating that tobacco plants transformed with the coat protein (CP) gene of tobacco mosaic virus (TMV) showed resistance to TMV infection. Since then, full-length, truncated and untranslatable constructs of CP gene sequences from a number of plant viruse have been transformed into many different plant species to engineer CP-mediated (CPM) resistance against viruses. Coat protein-mediated resistance has been successfully developed against a number of positive-stranded RNA viruses in the genera *Potyvirus, Cucumovirus, Ilarvirus, Tobravir*us, *Potexvirus, Tobamovirus, Tobravirus, Carlavirus,* and *Luteovirus* in many crop plants

including vegetables, fruits, cereals and forage crops (Baulcombe, 1994; Beachy, 1994; Hackland et al. 1994; Pappu et al. 1995; Fuchs and Gonsalves, 1997). Morever, several transgenic plants engineered for CPM resistance have already been commercialized, and many more have been evaluated in field trials for release (Fuchs and Gonsalves, 1997).

Use of viral sequences other than CP genes has been explored to engineer pathogen-derived resistance to viruses in plants. Plants were transformed with non-coding sequences from the 5' and 3' UTR of viral genomes (Nelson et al. 1993; Zaccomer et al. 1993) as well as satellite RNAs (Harrison et al. 1987) and D-RNAs (Kollar et al. 1993) to produce transgenic plants resistant to viruses. In addition, nonstructural genes encoding the protease (Maiti et al. 1993; Vardi et al. 1993), the cell-to-cell movement protein (Malyshenko et al. 1993) and the replicase (Carr and Zaitlin., 1993; Palukaitis and Zaitlin, 1997) have been used for PDR against a number of viruses in many crops. Although the results have been somewhat variable with different plant-virus systems, the use of non-structural genes, especially movement protein and replication associated proteins such as RdRp, is a promising strategy for developing virus resistance in transgenic plants (Beachy 1994; Pappu et al. 1995; Palukaitis and Zaitlin, 1997).

Replicase-mediated resistance

Virus resistance induced by expression of native or modified forms of replication-associated genes, such as the RdRp RNA polymerase of viruses in plants, is called replicase-mediated resistance (RMR). The first RMR was developed against bacteriophage Q , where expression of the modified replicase and a replicase binding site in the host generated bacteria which were resistant to the bacteriophage Q (Inokuchi and Hirasima, 1987). Replicase mediated resistance to plant viruses was first reported by Golemboski et al. (1990) who

transformed tobacco plants with the non-structural gene of TMV encoding the 54 kDa protein to determine the function of this protein. Transgenic plants expressing the 54 kDa protein were highly resistant to TMV infection. Since this first report of resistance to TMV in tobacco, RMR has been extensively explored for a number other plant RNA viruses using full-length and defective constructs of replicase genes. Defective constructs contain truncated replicase, lacking either the 3' or 5' terminus, and the conserved GDD motif of the RdRp domain as well as mutants constructed with one or two amino acid changes in the conserved GDD motif. Replicase-mediated resistance has been successfully developed for a number of viruses from ten different genera including *Alfamovirus*, *Bromovirus*, *Comovirus*, *Cucumovirus*, *Luteovirus*, *Potexvirus*, *Potyvirus*, *Tobamaovirus*, *Tombusvirus* and *Tobravirus* (Palukaitis and Zaitlin 1997).

In the first example of replicase-mediated resistance to TMV induced by the 54 kDa protein, plants expressing the 54 kDa protein were highly resistant to infection with virions and RNA from the U1 strain of TMV from which the sequence was derived (Golemboski et al. 1990). The resistance to TMV infection was observed in both transgenic plants and protoplasts derived from transgenic plants. Since the resistance was expressed at the single cell level in protoplasts, it was suggested that the resistance was mainly due to interference with viral replication (Carr and Zaitlin, 1991). It was reported that the cell-to-cell movement of the TMV RNA was also suppressed in the inoculated leaves of the resistant plants (Nguyen et al. 1996). Tobacco plants were also transformed with full-length replicase genes encoding the 126 and 183 kDa proteins and a mutant gene for the 126 kDa protein with a bacterial transposable element to develop RMR to TMV. Plants expressing the full-length 126 and 183 kDa proteins did not show resistance to TMV. However, plants expressing the mutant

126 kDa protein were resistant to TMV and several other tobamoviruses (Donson et al. 1993). *N. benthamiana* plants were transformed with both a wild-type and a truncated replicase gene encoding the 54kDa of another tobamovirus, pepper mild mottle virus (PMMV). Plants expressing wild-type 54 kDa showed two types of resistance response to PMMV infection. Some of the plants were susceptible to PMMV infection, but they were able to recover from the PMMV infection later, indicating a delayed induced resistance. Other transgenic plants showed a complete resistance to PMMV from the beginning of the infection, and no symptoms were observed on them, indicating a pre-established resistance (Tenllado et al. 1995). On the other hand, plants expressing the truncated 54 kDa protein were either highly resistant or susceptible to virus infection, and they did not show delayed induced resistance (Tenllado et al. 1996).

The replicase gene from cucumber mosaic virus (CMV) was mutated at the GDD motif by a deletion of 94 nucleotides or a truncation at C- terminus by a frameshift mutation. *N. tabacum* plants transformed with this replicase construct showed absolute resistance to high concentrations of virions and RNA from the homologous Fny strains of CMV (Anderson et al. 1992). The transgenic plants show resistance to strains from subgroup I, which are closely related to the Fny strain of CMV, and they were mostly susceptible or only partially resistant to some strains from CMV subgroups II (Zaitlin et al. 1994). The resistance induced by defective CMV replicase operated at the single cell-level (Carr et al. 1994) to inhibit virus replication and restrict cell-to-cell and long distance movement of the virus in transgenic tobacco (Carr et al. 1994; Hellwald and Palukaitis, 1995; Nguyen et al. 1996) and tomato plants (Gal-On et al. 1998). Although transgenic plants showed very limited cell-to-cell virus movement compared to nontransgenic plants, long distance movement of CMV was

completely inhibited by blocking the entry of the virus into the vascular system (Wintermantel et al. 1997). Analysis of transgenic plants containing the translatable and non-translatable replicase gene of CMV and examination of steady-state mRNA levels suggested that translatability of the transgene increases the effectiveness of replicase-mediated resistance to CMV (Wintermantel and Zaitlin, 2000).

Different forms of the alfalfa mosaic virus (AlMV) P2 replicase gene, including full-length and N-terminally truncated and modified constructs in which the conserved GDD motif was mutated to GGD, GVD , VDD or DDD, were used to engineer resistance to AlMV in tobacco (Brederode et al. 1995). No resistance was observed in transgenic plants expressing the full length, truncated and VDD mutants, however, complete or partial resistance to AlMV was achieved by the expression of the GGD, GVD, and DDD mutant replicase gene in tobacco. Analysis of transgenic plants showed that resistance was associated with the high level expression of mutant replicases (Brederode et al. 1995). In contrast, transgenic plants expressing full-length functional replicase from AlMV were not only fully susceptible to virus infection, but they were also capable of complementing replication of the mutant virus lacking its replicase gene (Taschner et al. 1991).

Replicase-mediated resistance was developed to three different potyviruses using different constructs of nuclear inclusion b (NIb) from potato virus Y (PVY), plum pox virus (PPV) and pea seed-born mosaic virus (PsbMV). *N. tabacum* plants transformed with full-length NIb gene and 3' and 5' truncted NIb genes conferred resistance to PVY infection, but the plants expressing NIb gene lacking the GDD motif did not show resistance to PVY. The resistance was specific to the strain from which the sequences were obtained, and plants were susceptible to even very closely related stains of PVY (Audy et al. 1993). *N. benthamiana*

plants expressing mutant NIb genes in which the GDD motif was changed to ADD or VDD were resistant to PPV infection. Plants carrying the NIb gene with the VDD mutation showed a delayed resistance response characterized by total recovery from the initial infection with PPV (Guo and Garcia., 1997). A similar type of resistance response was observed in transgenic pea plants expressing full-length NIb from PsbMV (Jones et al. 1998).

Transgenic tobacco plants with full-length and 5' truncated 166 kDa replicase of potato virus X (PVX) exhibited resistance to PVX, but plants expressing partial sequences of this gene encoding the nucleotide binding domain or the GDD motif were not resistant to PVX (Braun and Hemenway, 1992). The mutant replicase genes of PVX with single amino acid mutations in the GDD motif, GED, GAD or ADD as well as untranslatable sequence of the replicase gene did induce resistance to PVX in transgenic tobacco plants (Longstaff et al. 1993; Mueller et al. 1995). The transgenic plants resistant to PVX showed resistance only to a specific strain, and also low transgene RNA accumulation (Mueller et al. 1995).

A variable level of resistance has been achieved by expressing full-length replicase genes of several viruses including pea early browning virus (PEBV) (MacFarlane and Davies), cymbidium ringspot virus (CymRSV) (Rubino et al. 1993; Rubino and Russo, 1995), cowpea mosaic virus (CPMV) (Sijen et al. 1995), bromo mosaic virus (BMV) (Kaido et al. 1995), potato leaf roll virus (PLRV) (Kaniewski et al. 1995), and rice tungro bacilliform virus (RTBV) (Huet et al. 1999).

Mechanisms of replicase-mediated resistance

Virus resistance observed in transgenic plants expressing intact or modified viral replicase genes can be grouped into two broad categories. In the first category, the resistance is mediated by functional or mutant dysfunctional proteins which interfere with the replicase

enzyme complex and disrupt the viral replication. In the second category, expression of the protein is not required for resistance, and the resistance is mediated by the transgene RNA. Recent reports on two extensively studied RMR, TMV and CMV, showed that the resistance was contributed by both protein and RNA-mediated resistance mechanisms (Goregaoker et al. 2000; Wintermantel and Zaitlin, 2000). These findings showed that RMR may be complex in some systems, and that more than one factor can be responsible for the resistance phenotypes observed in transgenic plants.

Protein-mediated resistance

Protein mediated resistance (PMR) is characterized by a direct correlation between transgene protein and the degree of resistance. This type of resistance is usually effective against a broad spectrum of strains of the same virus, and even against some related viruses. Functional replicase expressed in a transgenic plant induces resistance to that virus if the level, time, and location of the expression in the cell interferes with replication, assembly, or movement of the virus (Carr and Zaitlin, 1993). Plants expressing the 54 kDa protein demonstrated a resistance response that is, in part, similar to this type of protein-mediated mechanism (Golemboski et al. 1990; Goregaoker et al. 2000). Functional inactivation of a gene by expression of a mutant form of the same gene is termed trans-dominant or negative dominant mutation. This inactivation can be achieved by competing for a factor, substrate, or a binding site or by formation of a non-functional complex of mutant and wildtype proteins (Herskowitz, 1987). It has been suggested that defective replicase expressed in transgenic plants may interfere with viral RNA replication by depleting host factors, or saturating the specific binding sites, or by forming a non-functional replication complex with replication-associated proteins of the virus (Carr and Zaitlin, 1993). Transgenic plants expressing

defective or mutant replicase of AlMV (Brederode et al. 1995), CMV (Carr et al. 1994; Wintermantel and Zaitlin, 2000) and TMV (Donson et al. 1993) all displayed resistance mediated by the mutant protein.

RNA-mediated resistance

Resistance responses determined by the transgene RNA transcript rather than the expression of the transgene protein product are termed RNA-mediated resistance. This type of resistance is characterized by detection of low steady-state levels of the transgene transcript in the transgenic plants and was first reported in tobacco plants transformed with the CP gene of tobacco etch virus (Lindbo and Dougherty, 1992). Similar RNA-mediated resistance was later reported in transgenic plants expressing mutant, translatable, and untranslatable replicase genes of PVX (Muller et al. 1995), PMMV (Tenllado et al. 1996), PPV (Guo and Garcia., 1997), and PsbMV((Jones et al. 1998). Expression of the protein is not required for RNA-mediated resistance, and in most cases of replicase-mediated resistance, the protein product of the transgene could not be detected in transgenic plants, thereby indicating that most replicase-mediated resistances are RNA-mediated (Baulcombe, 1995; Lommossoff, 1995; Baulcombe, 1996). Some transgenic plants display an instant resistance to virus infection; however, some transgenic lines display a recovery phenotype, in which an initial systemic viral infection was established, with the plants subsequently recovering from the initial infection and becoming completely resistant to virus infection (Lindbo, et al. 1993, Muller et al. 1995; Tenllado et al. 1995 Guo and Garcia., 1997).

The RNA-mediated and replicase-mediated resistances were shown to be very specific and effective only against the specific strain of the virus from which the transgene sequences were obtained or against closely related strains of the same virus with a high degree

of sequence homology (Audy et al. 1993; Zaitlin et al. 1994; Palukaitis and Zaitlin, 1997). Since this resistance is dependent upon extreme sequence similarity between the mRNA of the transgene and the inoculated virus, it is also called homology dependent resistance. Genetic analysis of RNA-mediated resistance in transgenic plants demonstrated that RNA-mediated resistance was related to post-transcriptional gene silencing (PTGS) in plants (Smith et al. 1994; English et al. 1996; Goodwin et al. 1996). Post-transcriptional gene silencing involves the reduction or suppression of gene expression by sequence specific degradation of the transgene mRNA (Stam et al. 1997). Although the actual mechanism of PTGS is not known, several models have been proposed based on extensive studies of PTGS in many systems. These include the RNA threshold model (Lindbo et al. 1993; Dougherty and Parks, 1995) and the aberrant RNA model (English et al. 1996;Wassengger and Pelissier, 1998). The observation of a virus induced resistance mechanism in non-transgenic plants which acts similarly to PTGS (Ratcliff et al. 1997; Ratcliff et al. 1999) and suppression of PTGS by viral pathogenicity determinants in silenced transgenic plants (Anandalakshmi et al. 1998; Brigneti et al. 1998) have suggested that PTGS is a natural defense mechanism of plants which was developed for protection against viruses (Ratcliff et al. 1999).

Genetic Engineering of Citrus

Genetic engineering is a promising approach for genetic improvement of woody plants, such as citrus, because their improvement by conventional breeding has been considerably limited by long juvenility period, heterozygosity, sterility, self- and cross-incompatibility. Currently commercialized *Citrus* genotypes can be made even more desirable by transforming them with specific genes from different sources to add new traits. As with other plants, genetic engineering of citrus requires the availability of genes for agriculturally important

traits and an efficient genetic transformation method to integrate the desired genes into the *Citrus* genome.

Development of an efficient genetic transformation technique has been attempted in different *Citrus* species using a variety of plant transformation methods. The first report of citrus transformation was the transformation of protoplasts of Trovita sweet orange by the direct DNA uptake method using polyethylene glycol (PEG) (Kobayashi and Uchimiya., 1989). Later, genetic transformation of protoplasts from rough lemon was also achieved using PEG-mediated direct DNA uptake (Vardi et al. 1990), and protoplasts from *C. reticulata* were successfully transformed using electroporation (Hidaka and Omura, 1993). To develop alternative methods for direct DNA uptake, suspension cell cultures from sweet orange calli were transformed by co-cultivation with *Agrobacterium tumefaciens* (Hidaka et al. 1990), and particle bombardment was used for transformation of embryogenic cells of tangelos, resulting in transgenic embryos (Yao, 1997). Thus, transgenic citrus plants have been obtained using PEG and electroporation-mediated direct DNA uptake, *Agrobacterium*-mediated and biolistic transformation of protoplasts, and cell cultures from embryogenic callus. However, these methods were very inefficient because of preparation of protoplasts or cell suspension cultures (Gmitter et al. 1992), and the regeneration of transgenic plants was difficult for most citrus species.

An *Agrobacterium*-mediated transformation method for citrus was developed using epicotyl segments. In this method, epicotyl segments from rootstock cultivars Carrizo citrange or Swingle citrumelo seedlings were co-cultivated with *A. tumefacience*, and shoots were produced from transformed segments by organogenic regeneration. Then, whole transgenic plants were obtained by rooting the shoots (Moore et al. 1992; Moore et al. 1993). This

method was later adapted and used for more efficient transformation of epicotyl segments from *P. trifoliata* (Kaneyoshi et al. 1994). To improve the original *Agrobacterium*-mediated transformation method for epicotyl segments, factors affecting the transformation and regeneration efficiency were studied in detail, and the method was optimized and used for the efficient transformation of citrange, lime and sour orange (Gutierrez et al. 1997).

A similar method having relatively higher transformation and regeneration efficiency was developed to transform pineapple sweet orange. Transgenic shoots regenerated from epicotyl segments were not rooted; instead they were grafted on *in vitro* grown young rootstock seedlings using a micro-shoot tip grafting technique and then re-grafted onto vigorous seedlings of Rough lemon in the greenhouse (Pena et al. 1995a; Pena et al. 1995b). This method was later optimized and used for the genetic transformation of lime (*C. aurantifolia*) (Pena et al. 1997) and citrange (Cervera et al. 1998). It also was reported that Washington navel sweet orange was transformed using this method, with a modification in the grafting stage in which transgenic shoots were directly micro-grafted to greenhouse grown seedlings of Carrizo citrange (Bond and Roose., 1998). Even though several citrus species have been successfully transformed and transgenic plants regenerated using these methods, the recovery of whole transgenic plants still requires use of complicated shoot tip or micro-grafting techniques.

Recently, a transformation protocol overcoming the difficulties associated with rooting of transgenic shoots in the original protocol was described and used for the first successful transformation and recovery of Duncan grapefruit plants (Luth and Moore., 1999). This method not only increased the efficiency of *Agrobacterium*-mediated transformation, but it

also provided a simple and efficient protocol for rooting transgenic shoots in a relatively short time of one to three weeks, (Luth and Moore., 1999).

Genetic transformation and regeneration of mature transgenic citrus plants was achieved using an *Agrobacterium*-mediated transformation of internodal segments from mature tissue of sweet orange, followed by shoot tip grafting. Several mature transgenic Pineapple sweet orange plants were obtained, and these plants flowered and bore fruit in 14 months (Cervera et al. 1998). An *Agrobacterium rhizogenes*-mediated transformation protocol was developed as an alternative method to *A. tumefaciences* and used to transform Mexican lime (Perez-Molphe-Balch and Ochoa-Alejo, 1998).

Currently, the *Agrobacterium*-mediated transformation methods using the epicotyl segments of seedling or juvenile plants is the most efficient method for producing transgenic citrus plants. This method has been useful for expressing reporter genes in different *Citrus* species, and it is now being used for transferring agriculturally important genes into citrus cultivars and relatives. Production of transgenic *P. trifoliata* plants expressing a synthetic gene encoding a human epidermal growth factor was reported in 1996 (Kobayashi et al. 1996). More recently, Carrizo citrange was transformed with the halotolerance gen, HAL2, isolated from yeast to improve salt-tolerance in citrus (Cervera et al. 2000). Furthermore, the CP gene from different isolates of CTV has been introduced into sour orange (Gutierrez et al. 1997) and Mexican lime (Dominguez et al. 2000) in attempts to develop pathogen-derived resistance to CTV. In addition, transgenic Duncan grapefruit plants expressing CP and other sequences from the CTV genome were produced, and they are now being evaluated for resistance to CTV (Febres et al. 2000).

CHAPTER 3
SEQUENCING AND ANALYSIS OF THE RNA-DEPENDENT RNA POLYMERASE GENE OF CTV ISOLATES

Introduction

Despite wide variation in morphology, genome organization and sequences in their structural proteins, all positive-stranded RNA viruses encode an RNA-dependent RNA polymerase (RdRp) (Goldbach., 1988). The RdRp functions as a catalytic subunit of viral replicase and is required for replication of the viral genome (Buck 1996). Comparative analysis of the amino acid sequences of the putative RdRp from positive-stranded RNA viruses reveals the presence of conserved sequence motifs (Kamer and Agros, 1984). Currently, eight conserved motifs have been identified in RdRps of RNA viruses, some of which also are present in other polymerases (Poch et al. 1989; Koonin, 1991). The conserved motifs of the RdRp correspond to the catalytic site for RNA polymerization (GDD motif), nucleoside triphosphate binding site, and the template and product binding sites (LKR motif) (Ishihama and Barbier, 1994). Inhibition of virus replication by specific point mutations in some of these conserved motifs demonstrated that the motifs are functionally important (Peters et al. 1994; Jablonsky and Marrow,1995; Davenport and Baulcombe, 1997). Since the RdRp is common in all positive-stranded RNA viruses and its amino acid sequence is conserved among different groups of viruses, the RdRp genes have been used for phylogenetic analyses and classification of positive stranded RNA viruses (Koonin 1991; Koonin and Dolja, 1993; Zanotto et al. 1996).

Sequence analysis of the CTV genome showed that a 56-kDa protein encoded by ORF1b is expressed using a +1 translational frameshift at the carboxy terminus of the polyptotein. This protein contained sequences with conserved motifs typical of RdRp of positive- stranded RNA viruses (Karasev et al. 1995). Comparison of the complete genome sequences of three CTV isolates revealed an unusual asymmetric sequence similarity along the genome in which the 3' half of the genome is more conserved with over 90% sequence identity, than the 5' half of the genome, where as low as 70% identity occurs (Mawassi et al. 1996; Yang et al.1999). Unexpectedly, these isolates showed as much as 25% sequence variation in their RdRp genes. Since the RdRp gene sequences from only three isolates were used for this analysis, better understanding of sequence variability in the RdRp gene of CTV requires more sequence information. In this study, the RdRp gene sequences from biologically and geographically different isolates of CTV were determined using a direct DNA sequencing approach, and the sequence variation and the phylogenetic relationships among CTV isolates were analyzed.

Materials and Methods

Virus Isolates

Citrus tristeza virus isolates T3, T30, T36, T66, SY568, B53, B165, B185, B249, and VT were obtained from the Collection of Exotic Citrus Diseases, in Beltsville, MD, USA. The biological characteristics of these isolates are summarized in Table 3-1.

Oligonucleotide Primers

Primers for PCR amplification were designed based on conserved regions located up- and down-stream of the RdRp genes of the three CTV genomic sequences in the GenBank (Accession No: U16304, U56902 and AF001623) (Figure 3-1). Based on the sequence

Table 3-1. Characteristics of the CTV isolates used in this study.

Isolates	Origin	Biological Characteristics				
		VC	SY	QD	SP-G	SP-O
T3	Florida	+	—	+	+	—
T30	Florida	+	—	—	—	—
T36	Florida	+	+	+	—	—
T66	Florida	+	+	+	—	—
3800	Florida	+	—	ND	+	—
B53	Japan	+	+	+	+	+
B185	Japan	+	+	+	+	+
B249	Venezuela	+	+	+	+	+
B165	India	+	+	+	+	+
T385	Spain	+	—	—	—	—
VT	Israel	+	+	+	—	—
SY568	California	+	+	—	+	+

VC= vein clearing on Mexican lime only. SY= Seedling yellows on sour orange seedlings. QD= Quick decline of scions grafted on sour orange rootstock. SP-G= Stem pitting on grapefruit scions. SP-O= Stem pitting on sweet orange scions. ND= Not determined.

information obtained by using these external primers, other internal primers were designed to complete the sequencing.

RNA Isolation and Complementary DNA (cDNA) Synthesis

Double-stranded RNA (dsRNA) was isolated from CTV-infected bark tissue using a CF11 cellulose-based protocol reported by Valverde et al. 1990. The cDNA was synthesized from dsRNA templates using primers CN306 or CN308 with Superscript II reverse transcriptase (GIBCO/BRL) according to the manufacturer's instructions.

Polymerase Chain Reaction (PCR)

Amplification of the RdRp region by PCR was performed using *Taq* DNA polymerase (Promega) in 50 l reactions containing 50 mM KCl, 10 mM Tris-HCl pH 9.0, 0.1% Triton X-100, 2.5 mM $MgCl_2$, 0.1 mM of each deoxyribonucleotide triphosphate (dNTP), 100 pmol of each primer, and 2-10 l of cDNA template. The mixture was incubated at 94 °C for 2.5 min for initial denaturation, and 94 °C for 1 min, 45 °C for 1 min, and 72 °C for 2 min for 40 cycles followed by one cycle at 72 °C for 10 min.

Sequencing of the RdRp Genes of CTV

PCR amplified RdRp genes were sequenced at the University of Florida DNA Sequencing Core laboratory using ABI Prism Dye terminator cycle sequencing protocols developed by Applied Biosystems (Perkin-Elmer) and using CTV RdRp specific primers. The flourescent-labeled extension products were analyzed on a Applied Biosystems Model 373 Stretch DNA Sequencer (Perkin-Elmer). Sequencing strategy and the primers used for sequencing are shown Figure 3-2 and Table 3-2, respectively.

Table 3-2. Sequences of the oligonucleotide primers used in this study.

Primers	Sequence	Orientation	Position[a]
CN305	5' GAATATAAGGGTAGTAAAGC 3'	Sense	9198-9218
CN306	5' GCAAACATCTCGACTCAACTACC 3'	Anti-sense	10881-10913
CN307	5' TTTACTGAGATGACGAACGCTG 3'	Sense	9898-9119
CN308	5' CGCGTCGAATTTTATGAGTCCC 3'	Anti-sense	11146-11167
CN309	5' TGTTTTGTACCGGACCCTTA 3'	Sense	10405-10424
CN310	5' GTACTCGCCTTCCATCCA 3'	Anti-sense	10081-10098

[a]The positions of the primers are indicated according to the genomic sequence of CTV strain T36.

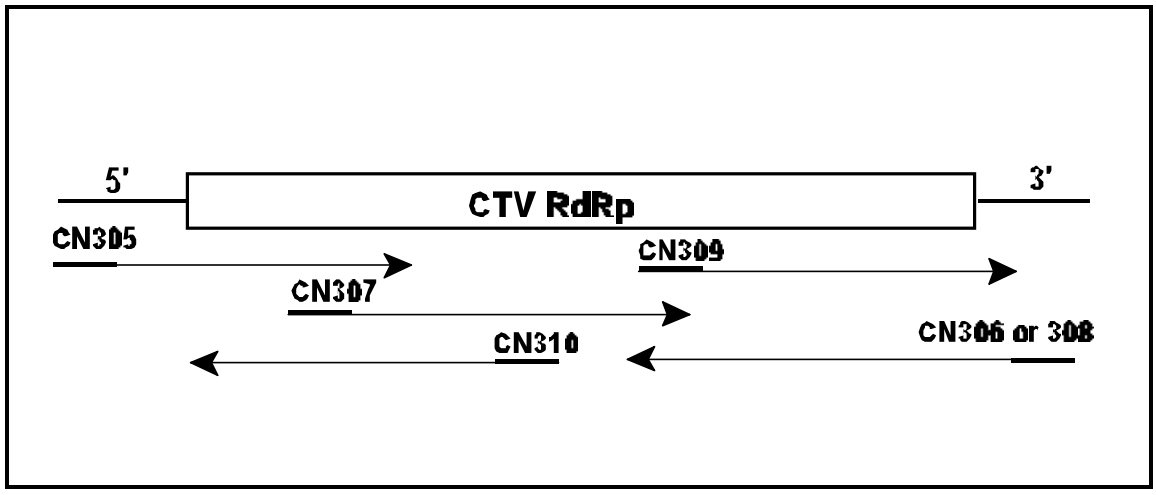

Figure 3-1. Schematic representation of the RdRp region of CTV and the strategy used for sequencing the RdRp gene. The black bars indicate the location of the primers and the lines with arrowheads indicate the length and direction of the sequence obtained from the specific primers.

Sequence Analysis

The RdRp nucleotide sequences were assembled and translated using the Genetics Computer Group (GCG). Multiple sequence alignments were generated in the Align X program of the Vector NTI suite. Phylogenetic analyses were done using the Diverge module of GCG which makes codon-by-codon comparison of aligned protein coding sequences and estimates the number of synonymous and non synonymous substitutions per site (Li, 1993). The phylogenetic tree was generated in the GrowTree module of GCG using Kiamura two-parameter algorithms with the neighbour-joining and UPGMA methods.

Results and Discussion

Primers designed based on conserved sequences located up- and downstream of the RdRp gene of three different isolates of CTV were used to amplify the RdRp genes from a number of biologically and geographically different isolates of CTV. The RdRp sequence of CTV isolates T30, B53, B165, B185, B249 and 3800 were determined using direct DNA sequencing of the ORF 1b region. Two different RdRp sequences, designated as T2K and T38K, were obtained from CTV isolate 3800, which possibly contains two distinct virus populations (Manjunath et al. 2000). In addition to these sequences, the RdRp sequences of CTV isolates T36 (Karasev et al. 1995), SY568 (Yang et. al.,1997), VT (Mawassi et al.

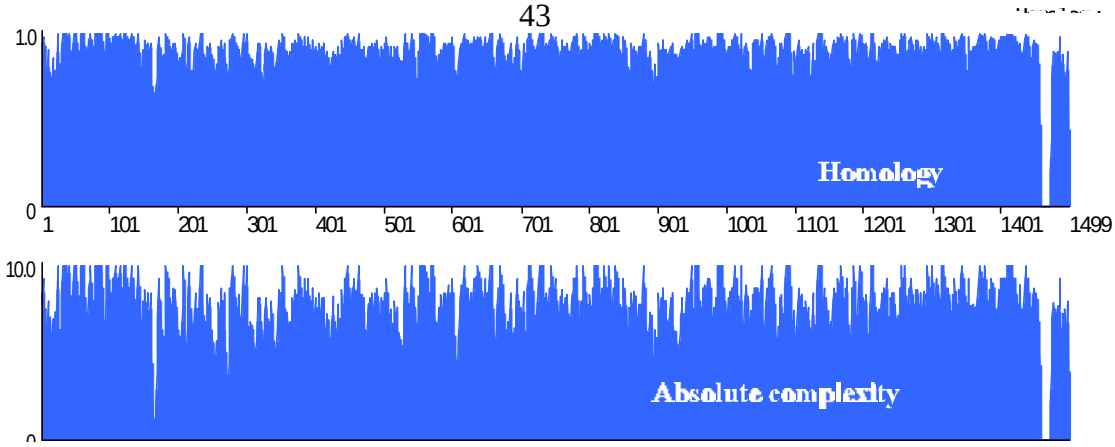

Figure 3-2. Homology (Top) and absolute complexity (Bottom) graphs of the nucleotide sequences of the RdRp gene from 10 biologically and geographically different isolates of CTV. The graphs were generated using the Align X program of Vector NTI Suite. The homology graph displays alignment quality based on the total similarity values (1, 0.5 and 0.2 for identical, similar or weakly similar, respectively) for the residues at a given position. The absolute complexity graph displays a statistical significance profile of the alignment calculated as the sum of all pair-wise substitution scores at a given alignment position. The 5' end of the RdRp gene is to the left.

Table 3-3. Percentage of nucleotide sequence identity of the RdRp genes from different isolates of CTV. The calculations were made using Clustal W. The most similar isolates are shown in blue and the most different isolates are shown in red

Isolates	B53	B249	VT	SY568	T30	B165	T36	3800	T385	T2K	T38K
B185	99	98	96	91	91	85	79	83	91	82	78
B53		98	96	91	91	85	79	83	91	82	85
B249			96	90	91	85	79	83	91	82	84
VT				89	89	84	79	82	90	82	84
SY568					91	81	75	**79**	91	**79**	81
T30						85	78	83	**99**	82	85
B165							79	83	85	81	86
T36								85	78	89	78
3800									83	90	89
T385										82	85
T2K											80

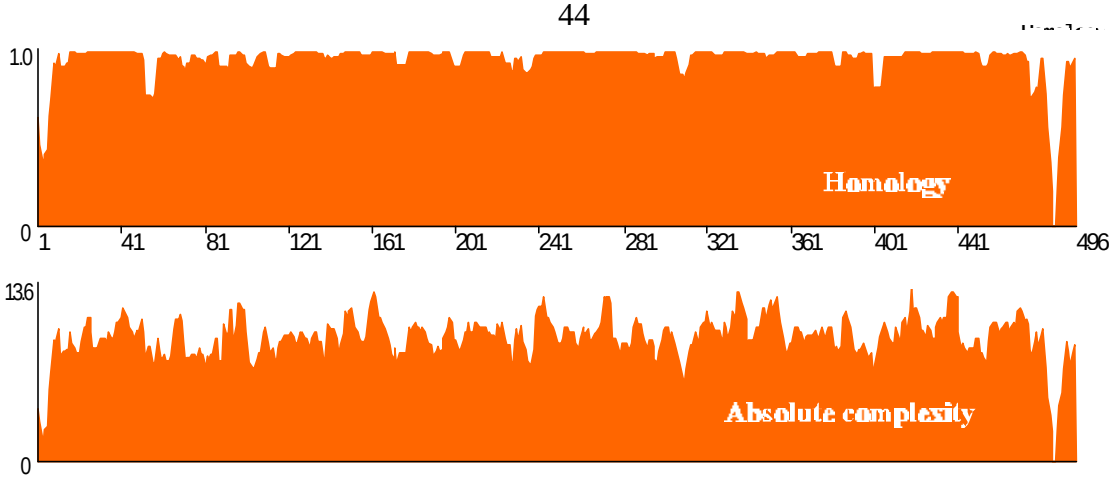

Figure 3-3. Homology (Top) and absolute complexity (Bottom) graphs of deduced amino acid sequences of the RdRp gene from10 biologically and geographically different isolates of CTV. The graphs were generated using the Align X program of Vector NTI Suite. The homology graph displays alignment quality based on the total similarity values (1, 0.5 and 0.2 for identical, similar or weakly similar, respectively) for the residues at a given position. The absolute complexity graph displays a statistical significance profile of the alignment calculated as the sum of all pair wise substitution scores at a given alignment position. The 5' end of the RdRp gene is to the left.

Table 3-4. Percentage of and amino acid sequence identity of the RdRp genes from different isolates of CTV. The calculations were made using Clustal W. The most similar isolates are shown in blue and the most different isolates are shown in red.

Isolates	B53	B249	VT	T30	B165	T36	3800	T385	T2K	T38K
B185	99	98	97	96	94	90	90	96	93	92
B53		99	96	95	93	89	90	95	92	92
B249			96	95	94	90	90	95	93	92
VT				94	91	87	88	94	90	90
T30					93	89	90	**100**	92	92
B165						88	90	93	92	92
T36							91	89	94	88
3800								90	95	93
T385									92	92
T2K										90

including B53, B185, B249 and VT exhibited 96 to 99% sequence identity to each other (Table 3-3). Comparative analyses of the deduced amino acid sequences of the RdRp genes confirmed that T36 was the most diverse isolate with as much as 13% or 65 amino acid differences in the RdRp gene (Table 3-4). On the other hand, the RdRp of two mild isolates,T30 from Florida and T385 from Spain, were 100% identical and only a few amino acid differences were identified among the RdRps of B53, B185 and B249, all SP-inducing isolates from Spain, Japan and Venezuela, respectively. The B53 isolate reportedly was originally found in an early satsuma mandarin illegally imported to Spain from Japan (Ballester-Olmos et al.1988). This implies that B53 and B185 might have the same origin (Table 3- 4). The remaining isolates had 5 to10% variation in the amino acid sequences of their RdRp genes. The isolates sequenced in this study (B53, B165, B185, B249 and T30) were more similar to each other than the previously sequenced isolates (T36, VT and SY568). This may be due to differences in the sequencing methods used. While T36, VT and SY568 were sequenced from clones, a direct DNA sequencing method was used in this study. One of the major advantages of direct sequencing compared with sequencing of cloned DNA is that it allows rapid determination of the major sequence of heterogenous virus populations (Odeberg et al. 1995). The nucleotide reported at a specific position can be the additive result of several minor isolates present in the population, identical at a specific position but different at other positions. The major sequences obtained by direct sequencing is the consensus sequence of heterogenous virus populations present, but is not necessarily the sequence of the most abundant strain (Odeberg et al. 1995). In contrast, sequences obtained from cloned DNA sequencing do not represent the population, but individual

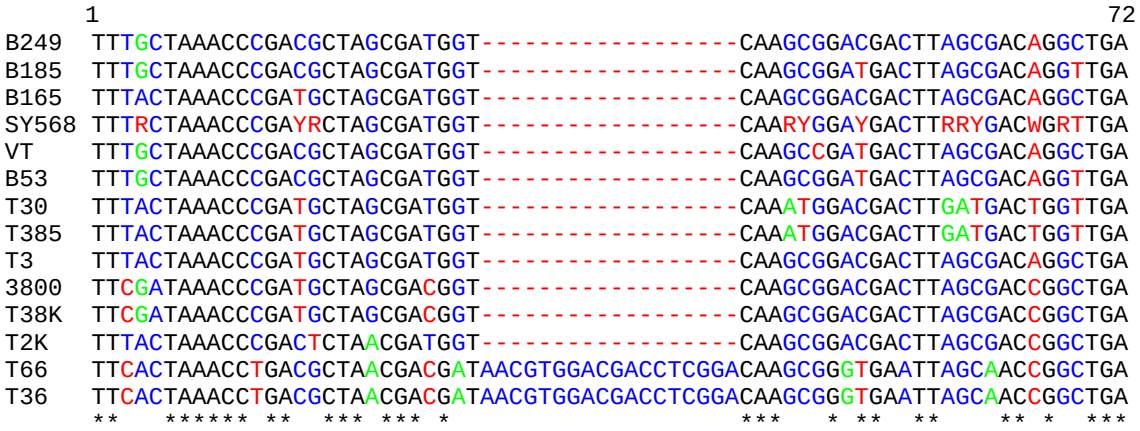

Figure 3-4. Multiple sequence alignment of the 3' region of the RdRp genes of CTV isolates showing the 18 base insertion in some isolates. The alignments was produced using AlignX module of Vector NTI. The asterisks indicates identical nucleotide in all isolates and dashes indicate missing nucleotide sequence. Number shows the position of the nucleotide in the genomic sequence of CTV strain T36.

strains, possibly the most abundant strain in a heterologous virus population. In order to get a representation of a virus population, a large number of clones would have to be sequenced (Odeberg et al. 1995). Multiple alignment of the RdRp genes also revealed an 18-nucleotide in frame insertion close to the 3' end of RdRp gene of T36, a QD isolate from Florida (Figure 3-4). This insertion was not found in the RdRp gene of any of the ten isolates previously sequenced or determined in this study. To identify other isolates having the 18 nucleotide insertion in their the RdRp gene, the 3' half of the RdRp gene of two other CTV isolates from Florida, T66 and T3, were amplified and directly sequenced from the PCR products. The insertion sequence was found in T66, which is a QD inducing isolate similar to T36, but it was not present in T3 which has a different biological activity (Figure 3- 4). In order to screen more CTV isolates for the presence of the insertion sequence, a PCR primer specific to the insertion sequence was designed. More than 20 CTV isolates from the Collection of Exotic Citrus Diseases and 10 isolates from Spain were tested by PCR using the insertion specific

primer and an internal primer specific to a region conserved in all CTV RdRp genes. Among these isolates, only one QD inducing isolate from the collection contained the insertion sequence. Although all three isolates with the 18-nucleotide insertion sequence in their RdRp gene do induce QD, other QD inducing isolates tested by PCR did not have the insertion, thus indicating that the insertion may not be related to the biological characteristics of CTV isolates.

The RdRp is conserved in all positive-stranded RNA viruses, and it has been used to determine the evolutionary relationships among the positive- stranded RNA viruses, and also in their classification (Koonin, 1991; Koonin and Dolja, 1993; Zanotto et al. 1996; Hong et al. 1998). The RdRp gene also has been used for phylogenetic analyses of isolates of a number of animal and plant RNA viruses (Arankalle et al. 1999; Hitomoto et al, 2000; Bousalem et al. 2000). In this study deduced amino acid sequences of the RdRps were analyzed to determine the evolutionary relationships among CTV isolates with diverse biological properties and different geographical origin.

On the basis of phylogenetic analysis of the RdRp genes from different isolates of CTV, two distinctly branched clusters could be identified. One of the main clusters (I) consisted of only two isolates, T36 and T2K, from Florida. The second main cluster (II) contained all other isolates of CTV and was divided into two sub-clusters (Figure 3-5). The isolates 3800, T38K and B165 were grouped together in one sub-cluster (IIA) and the remaining isolates grouped into sub-cluster IIB, which is further divided in to two different branches (IIB-1 and IIB-2) (Figure 3-5). The clusters clearly are not related by the

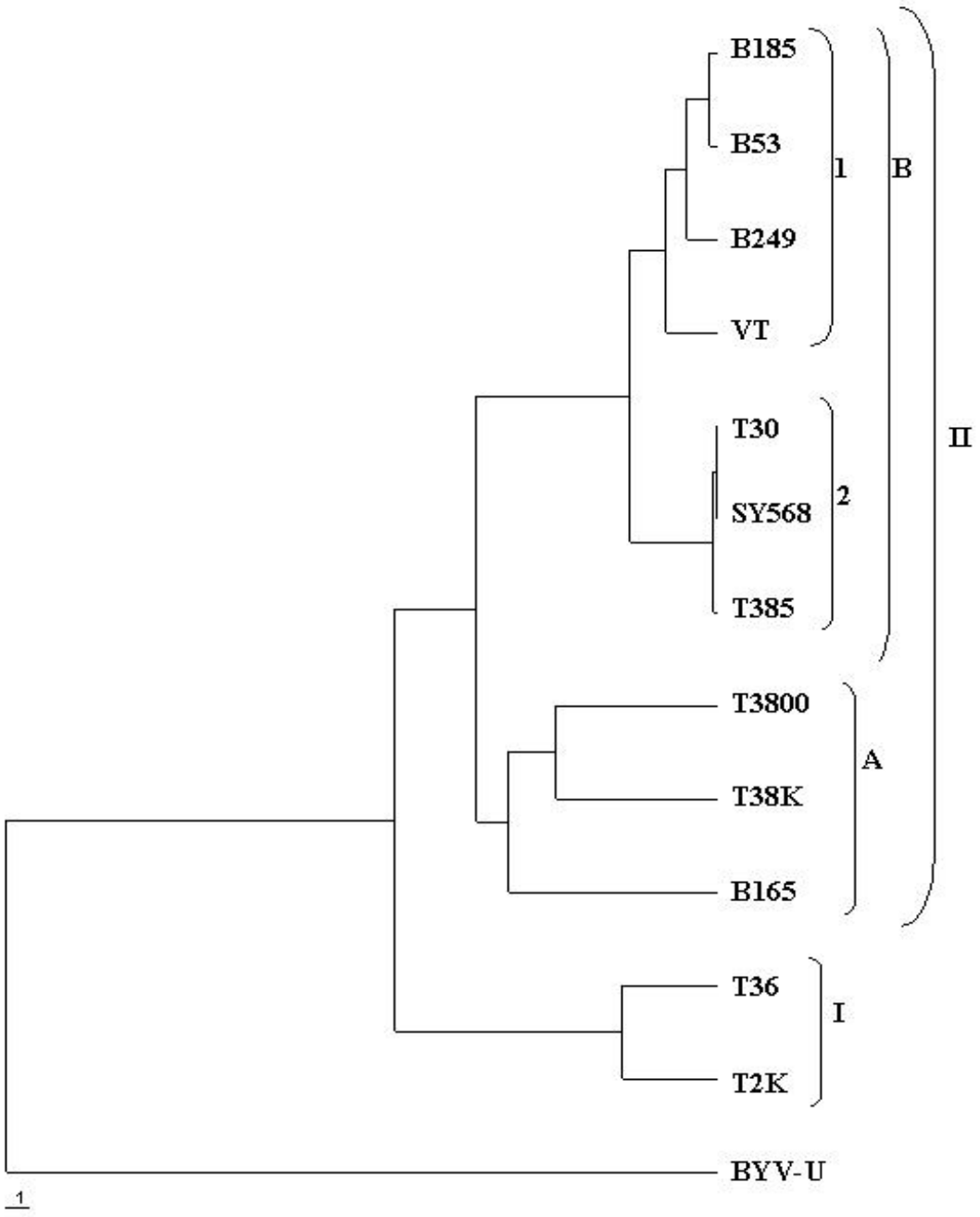

Figure 3-5. A phylogenetic tree showing relationships among CTV isolates based on their RdRp genes sequences. The phylogenetic tree was generated by GCG's Diverge and GrowTree programs using sequence alignments and matrix of evolutionary distances determined by the number of synonymous and non synonymous substitutions per site in the protein coding sequences. Biological characteristics and geographical origins of the isolates are shown in Table 3-1. The RdRp gene of BYV was used as the out group.

geographical origin of the isolates. However, there is some association with the biological properties of the isolates. Cluster I contains the known QD isolate from Florida, T36, and also T2K, a sub-population of isolate 3800, which induces SP on grapefruit but has not been tested for QD. It is possible that T2K may be the QD inducing component of isolate 3800. Cluster IIA consists of the known SP isolates B165 and 3800 from India and Florida, respectively. The presence of T38K, the other sub-population of isolate 3800, in this group implies that T38K may be the SP-inducing component of the isolate 3800. The cluster IIB-1 contains two well characterized mild isolates of CTV, T30 from Florida and T385 from Spain, and a known seedling yellows isolate SY568. Comparative analyses of genome sequences of SY568 and other isolates of CTV suggested that the SY568 genome may have resulted from RNA recombination between two CTV genomes, one of which was almost identical to T385 (Vives et al. 1999). Thus, it is possible that the cluster IIB-1 actually contains only the mild isolates of CTV. The cluster IIB-2 may be considered as the second SP group, since it consists of well characterized SP isolates from different regions of the world. It can be further inferred from this phylogenetic information that there are two lineages of CTV: the QD lineage, and the second lineage which later diverged to mild and SP lineages. Although isolates with similar biological activities grouped together in the phylogenetic analyses, the results are not conclusive because of the limited number of isolates analyzed in this study.

Conclusions

Highly conserved regions of the CTV genome were used to design PCR primers for non-selective amplification of the RdRp gene from very distinct isolates of CTV. These would also be used for characterization of even more variable regions of the CTV genome. Direct sequencing of PCR products is an effective method for identification of consensus

sequences found in a virus population. It also is easier, faster and more economical than sequencing from the cloned cDNAs. The RdRp genes of biologically and geographically different isolates of CTV are conserved and related in their nucleotide and amino acid sequence. The most obvious difference among the RdRp genes of CTV isolates is the presence of an 18-nucleotide in-frame insertion sequence close to the 3' end of the RdRp genes of isolates T36 and T66, both QD isolates from Florida. At this point, the insertion sequence appears to be rare and is not associated with all QD isolates.

On the basis of comparative sequence analyses, the QD isolate T36 from Florida was the most distinct isolate, and the RdRp genes of some CTV isolates with similar biological activity have a significantly higher level (98 to100%) of sequence identity, indicating that they are more closely related at the molecular level. Since CTV isolates show unusual and asymmetric sequence relationships along their genomes, comparison of the RdRp genes sequences may provide the most straight forward method for phylogenetic analysis. Based on these phylogenetic analyses of their RdRp genes, CTV isolates can be divided into four distinct groups. Three of these groups are interrelated, suggesting that they diverge from the same lineage. However, the other group is more distantly related to these groups indicating that it may be from a different lineage. Although it is not conclusive, the groups appear to contain biologically similar isolates.

CHAPTER 4
CLONING AND EXPRESSION OF THE RNA-DEPENDENT RNA POLYMERASE OF CTV IN *Escherichia coli*

Introduction

All positive-stranded RNA viruses use an RNA dependent RNA polymerase (RdRp) for their replication. The RdRp is the catalytic subunit of the viral replicase complex which includes other viral and host proteins. Therefore, they are absolutely required for the replication of the virus in the host cell (Buck, 1996). Although the RdRps are universally conserved among different groups of RNA viruses, they express their RdRps using a variety of expression mechanisms. In some viruses, the RdRp is produced as a single peptide, but in other viruses, it is expressed as a polyprotein containing RdRp and one or more other viral proteins. The expression of the RdRp is controlled and regulated by different mechanisms in different viruses by proteolytic processing, translational readthrough or frameshifting (Gallie, 1996). The RdRp of CTV is encoded by ORF1b. It overlaps with ORF1a which encodes two proteases and the other replication associated proteins, a methyltransfrase and a helicase. It is proposed that the CTV RdRp is expressed by a + 1 translational frameshifting and possibly requires proteolytic processing to liberate it from the polyprotein encoded by ORF1a\1b (Karasev et al. 1995).

Isolation of viral RdRps from infected host cells is very difficult because they are expressed and accumulated at very low levels. Consequently, most viral RdRs have been identified based on sequence analysis of conserved domains (O'Reilly and Kao., 1998).

However, the catalytic activity of RdRp has been demonstrated for a number of animal and plant viruses in partially purified extracts isolated from infected host cells (Graaff and Jaspar. 1994). Among the plant viruses, the partially purified extract of RdRps of turnip yellow mosaic virus (TYMV) and brome mosaic virus (BMV) have been well-characterized and used in *in vitro* studies of viral replication. Development of these *in vitro* replication systems using their RdRps revealed information on the mechanism and regulation for the replication of these and similar viruses (Singh and Dreher, 1997; Sun and Kao, 1997; Siegel et al. 1998). On the other hand, for most viruses the isolation of partially purified active RdRp from the infected plants was unsuccessful due to host characteristics, low expression level, and/or poor solubility of the purified RdRp itself (Graaff and Jaspar. 1994).

Difficulties associated with the purification of the RdRp from the infected host cell may be overcome by expression of the RdRps in *E. coli*. Enzymatically active RdRps of poliovirus (Rothstein et al. 1998), hepatitis C virus (Behrens et al. 1996; Lohmann et al. 1997), tobacco vein mottling virus (Hong and Hunt, 1996) and bamboo mosaic virus (BaMV) (Li et al. 1998) were expressed in *E. coli*, and their function and catalytic activity were characterized. Some other viral RdRp were expressed in *E. coli* and the products were used for production of antibodies specific to viral RdRps. These antibodies were later used for detection, identification and functional dissection of RdRps of RNA viruses in the infected host tissue (Hayes et al. 1994; Scholthof et al. 1995).

In this study, the RdRp gene of CTV isolate T36 was cloned and expressed in *E. coli* and polyclonal antibodies to the expressed protein were produced in order to detect and localize the RdRp of CTV in the infected citrus tissue.

Materials and Methods

Cloning of the CTV RdRp Gene

The cDNA specific to the RdRp region of CTV isolate T36 was synthesized from dsRNA templates using primer CN257 (5' *CTACTCGAG*ATCTATCAATCGATCAGCC GGTT 3') with *Superscript* II reverse transcriptase (GIBCO/BRL). The RdRp gene was amplified from cDNA by PCR using Taq DNA polymerase with the primers CN256 (5' *TGTAAGCTT**ATG***GAGACACCGCCCCTCCT 3') and CN257. These primers were designed based on published sequence information for isolate T36 (Karasev et al. 1995), and since the RdRp gene does not have an initiation codon, an ATG codon was incorporated into the plus-sense primer CN256. In addition, the specific recognition sequences for the restriction enzymes *Hind* III and *Bgl* II (underlined sequences) were incorporated into the primers CN256 and CN257, respectively, to facilitate the cloning into the expression vector. The amplified RdRp gene was first cloned into the pGEM-T cloning vector (Promega) using the T-A cloning method. The *Hind* III/*Bgl* II fragment of the pGEM-T clone containing the RdRp gene was sub-cloned into the pETh-3c expression vector (Invitrogen), and the resulting plasmid was designated as pETh3c-CTVRdRp. The presence and the integrity of the CTV RdRp ORF in the pETh3c vector was confirmed by sequencing.

Expression of the CTV RdRp

Escherichia coli strain BL21 was transformed with the pETh3c-CTVRdRp, and protein expression was induced by 0.4 M isopropyl thio- -D-galactoside (IPTG) at 37 ^{0}C for 3 hrs. The expressed protein was separated from other bacterial proteins on a 10 or 12% SDS-polyacrylamide gel by electrophoresis (SDS-PAGE) and stained with Coomassie brilliant blue G-250 (Sambrook et al. 1989)

Production of Polyclonal Antibodies Specific for the CTV RdRp

The recombinant protein was produced in large scale and separated from other bacterial proteins by SDS-PAGE and stained with 0.3 M $CuCl_2$ to visualize the bands . The protein band was excised from the gel, and the gel slice destained in 0.25 M EDTA/ Tris-HCl, pH 9.0 (Lee et al. 1987). The protein in the gel slice (about 5-7.5 mg) was used for production of polyclonal antibodies in a rabbit by injecting about 1.0 -1.5 mg of expressed protein into the rabbit weekly for four weeks (Cocalico Biologicals Inc.). Antiserum was collected after the forth injection and tested for specificity to the expressed protein by Western blot analysis. To increase the antiserum titer, one more injection with (about 2.5 mg) more protein was performed after the fourth collection of antiserum.

Western Blot Analysis

Total protein was extracted from *E. coli* strain BL21 with or without the CTV RdRp gene and from CTV infected and healthy citrus tissue. The extracts were separated on 10-12 % SDS-PAGE and transferred to nitrocellulose membranes using a semi-dry blotter (Bio-Rad). The membranes were probed with different dilutions of antiserum raised against the expressed CTV RdRp followed by detection with alkaline phosphatase (AP) or horseradish peroxidase (HRP) conjugated anti-rabbit antibody. The membrane blots were developed using the BCIP and NBT colorimetric system for the AP and the Super Signal™ chemiluminescent substrate system (Pierce) for location of HRP.

Cell Fractionation Assays

Cell fractions of healthy and CTV- infected citrus tissue were prepared by differential centrifugation using a previously described protocol (Godefroy-Colburn et al. 1986). The cell

fractions were analyzed on 10-12% SDS-PAGE followed by Western blot and chemiluminescent detection system.

Results and Discussion

Expression of CTV RdRp in *E. coli*

Expression of CTV RdRp was induced in *E. coli* strain BL21 transformed with the pETh3c-CTVRdRp plasmid by addition of isopropyl thio- -D-galactoside (IPTG). CTV RdRp was expressed as a fusion protein with an estimated molecular weight of about 60 kDa, which contains about 2 kDa from the vector and 57 kDa from the CTV RdRp ORF. Separation and analysis of bacterial proteins by SDS-PAGE revealed a high level of expression of the 60 kDa protein only in the induced bacterial cells containing the pETh3c-CTV RdRp plasmid. The protein was not expressed in non-induced cells with the same plasmid or in induced cells containing the pETh3c vector alone (Figure 4-1). Since the expression of the protein was specifically induced and its size corresponded to the expected size, it was concluded that this protein was the CTV RdRp.

The induction of the fusion protein was evaluated periodically starting 30 min after addition of IPTG. The expression of the protein increased continuously over time and reached maximum at 3 hr after induction at 37 ^{0}C (Figure 4-2). The purification of the proteins from bacterial cells indicated that the expressed RdRp was not soluble, and was found only in the insoluble fractions of the *E. coli* cells. (Figure 4-3). To produce soluble CTV RdRp, expression was induced at lower temperatures such as 30 and 35 ^{0} C, and several different solvents such as urea were used to solubilize the recombinant CTV RdRp.

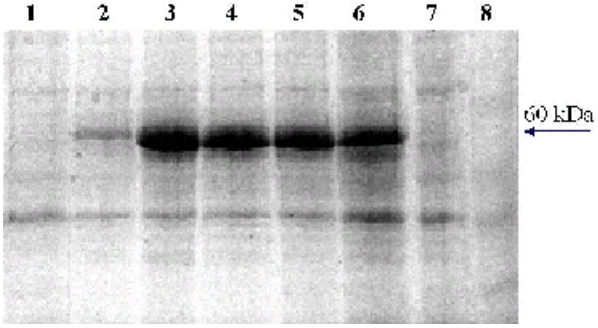

Figure 4-1. Induction of the expression of the CTV RdRp in *E. coli* by IPTG. Lanes 1and 7 induced and non-induced *E. coli* BL21 carrying pETh3c vector alone, respectively. Lane 2 is non-induced *E.coli* BL21 with the pET3c-CTVRdRp. Lanes 3-7 induced *E.coli* BL21 with pETh3-CTVRdRp; and lane 8 is molecular weight markers.

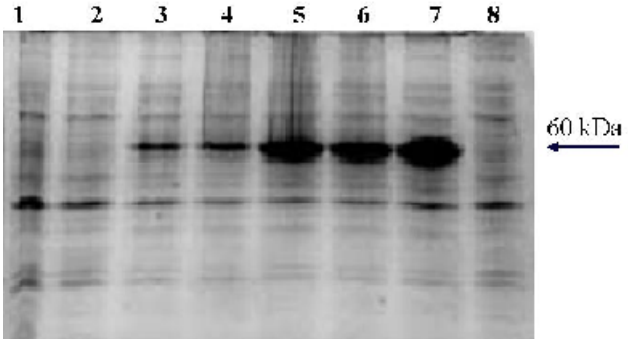

Figure 4-2. Time course study of expression of the CTV RdRp in *E. coli* after the induction by IPTG. Lanes 1and 2 are induced and non-induced *E. coli* BL21 carrying the pET3c vector alone, respectively. Lane 3 is non-induced *E.coli* BL21 with the pETh3c-CTVRdRp. Lanes 4-7 are induced *E. coli* BL21 with pETh3c-CTVRdRp at 30 min, 1, 2, and 3 hours after the induction by IPTG.

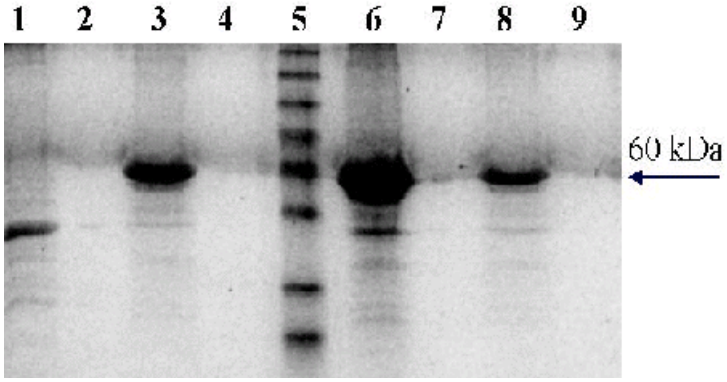

Figure 4-3. Accumulation of the expressed CTV RdRp in cellular fractions of *E.coli* BL21. Lanes 1 and 2 are insoluble and fractions, respectively, of *E. coli* BL21 carrying the pETh3c vector alone. Lanes 3, 6 and 8 are the insoluble fractions and lanes 4, 6 and 9 are soluble fractions of *E. coli* BL21with the pETh3c-CTVRdRp. Lanes 3-4, 6-7 and 8-9 were grown at 35, 37 and 30 ^{0}C, respectively.

However, the protein could not be solubilized and always remained in the insoluble fractions of the cell (Figure 4-3).

Production and Testing of Polyclonal Antibodies to the CTV RdRp

Polyclonal antibodies to the CTV RdRp were raised by injecting a rabbit with a total of 7.5-10 mg recombinant RdRp. After four weekly injections, antiserum was collected and tested for specificity by Western blot analysis. The antiserum reacted strongly with the 60 kDa protein expressed in bacterial cells containing the pETh3c-CTV RdRp plasmid, but not with proteins in cells carrying the pETh3c plasmid alone (Figure 4; lanes 1-4). A weak reaction also occurred with two other smaller proteins in bacterial cells containing the pETh3c plasmid with or without the CTV RdRp gene (Figure 4; Lanes 1-4). These results indicated that the antiserum was specific to the expressed CTV RdRp but that it also cross reacts with two abundant bacterial proteins. Different dilutions of the expressed CTV RdRp tested by Western blot analysis to determine the sensitivity of the antiserum showed that a 1:10,000 dilution of the antiserum could detect up to a 1:1000 dilution (about 10-100 ng) of the expressed protein (Figure 4; Lanes1-4). However, best results were obtained using 1:1000 dilution of the antiserum.

Detection and Sub-Cellular Localization of the CTV RdRp in CTV-Infected Citrus Tissue

To detect the expression of RdRp in the CTV-infected tissue, total protein from infected and healthy citrus bark tissue was separated by 10-12% SDS-PAGE, transferred to a nitocellulose membrane and probed with 1:1000 dilution of the antiserum to the 60 kDa recombinant protein. A non-specific reaction with a protein of about 55 kDa was detected in both infected and heathy tissue (Data not shown). This non-specific reaction was eliminated by cross-absorbing the antiserum first with a healthy tissue extract before using it to probe the blot. No specific reaction with the antiserum was detected in the healthy or infected tissue

using anti-rabbit antibody conjugated with AP followed by colorimetric detection with BCIP and NBT. However, a reaction with a 50 kDa protein in CTV-infected tissue extract, which was not present in the healthy tissue extarct, was observed using the more sensitive detection method involving anti-rabbit antibodies conjugated with HRP followed by chemiluminescent detention system (Figure 5; Lanes 3 and 4). The size of the protein does not correspond to the size of the putative protein product of the CTV ORF1b, expected to be 57 kDa, or the CTV polyprotein which is about 400 kDa. Although the detected protein band was discreet and solid, the reaction was weak and could only be detected when the gel was overloaded (using 25 l of extract compared to of 10 l) with the freshly prepared extract from the infected tissue. The difficulty detecting RdRp in the infected tissue could be due to a low level of expression of the CTV RdRp *in vivo*. In general, since the RdRp is a catalytic protein for RNA viruses their RdRps are expressed at low levels and could not even usually be detected in the infected tissue.

To determine the sub-cellular localization of CTV RdRp in the infected citrus tissue, fractionated preparations from infected and uninfected tissue extracts were analyzed by Western blot analysis using antibodies specific to recombinant RdRp of CTV. A band of about 50 kDa was detected in the membrane and cytoplasmic fractions of the CTV-infected tissue (Figure 6; lanes 4 and 8). However, no reaction was observed with any fractions of healthy tissue (Figure 6; Lanes 1, 3, 5, and 9). The band reacting with the antiserum was present in the cytoplasmic fraction, but it accumulated predominantly in the membrane fractions of the infected cells. Although the size of the protein detected in the infected tissue and some of its fraction is smaller than the expected size, of the specificity of the reaction

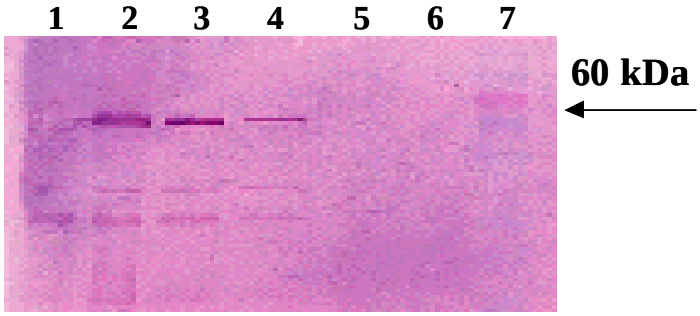

Figure 4-4. Western blot analysis of the CTV RdRp expressed in *E. coli*. Lane 1 is the pETH-3c vector without the RdRp as the negative control. Lanes 2, 3, 4, 5 and 6 are 1:10, 1:100, 1:1,000, 1:5,000 and 1:10,000 dilutions of the expressed protein, following purification from *E.coli*, respectively. Lane 7 is pre-stained protein markers.

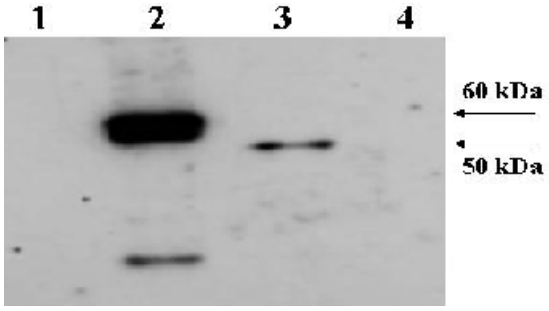

Figure 4-5. Detection of CTV RdRp in CTV-infected citrus tissue by Western blot analysis using antiserum prepared to the CTV RdRp expressed in *E. coli*. Lane 1 is CTV RdRp expressed in *E. coli* as the positive control. Lanes 2 and 4 are the negative controls from the extract of *E. coli* containing the pETH-3c without the RdRp and extracts of healthy citrus tissue, respectively. Lane 3 is the extract from citrus tissue infected with T36 isolate of CTV.

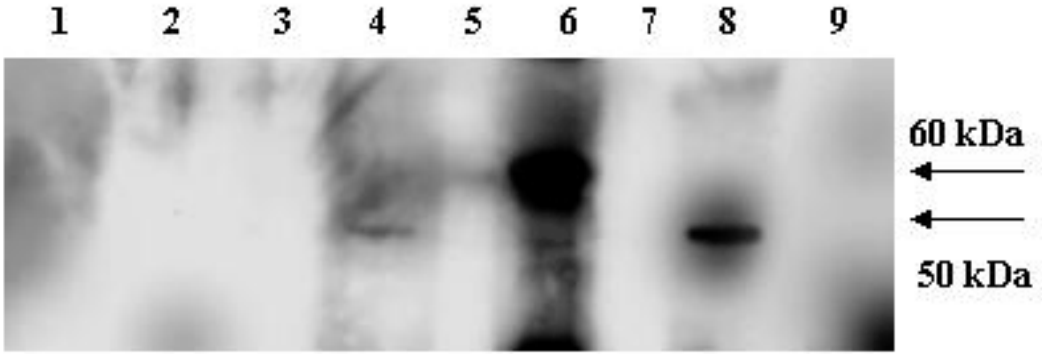

Figure 4-6. Sub-cellular localization of the CTV RdRp by Western blot analysis using antiserum prepared to the CTV RdRp expressed in *E. coli*. in citrus tissue infected with the T36 isolate of CTV. Lanes 1, 3, 5, and 9 are nuclear, cytoplasmic, cell wall and membrane fractions of tissue, respectively. Lanes 2, 4, 7 and 8 are nuclear cytoplasmic, cell wall and membrane fractions of citrus tissue infected with the T36 isolate of CTV, respectively. Lane 6 is the expressed protein as a positive control.

with the antibody and sub-cellular location where the protein was detected, indicate that the detected protein is likely the RdRp of CTV.

The accumulation of the CTV RdRp in the membrane fractions of the infected cells is consistent with previous reports that RdRps of most RNA viruses are membrane associated (Graaff and Jaspars, 1994). In addition, the detection of the BYV helicase, which interacts with RdRp during viral replication, in the membrane fractions also supports the suggestion that the CTV RdRp is predominantly associated with membrane fractions of the infected tissue.

It is possible that the RdRp could be processed and cleaved from the CTV polyprotein by a protease encoded by the virus or the host. Recently, it was reported that the helicase and methyltransferase domains were detected in BYV infected tissue as individual proteins indicating that these proteins were cleaved by the processing of the polyprotein (Erokhna et al. 2000). A host cystein-like protease involving the processing of the RdRp of *Leishamania* virus from its polyprotein precursor has already been identified (Young-Tae., 1997). Even though this virus has a dsRNA genome that is taxonomically unrelated to CTV, the expression of the RdRp gene using the + 1 translational frameshifting mechanism is common to both viruses.

Conclusions

The RdRp gene of T36, a QD isolate of CTV, was successfully expressed in *E.coli*, and a polyclonal antiserum specific to the RdRp was produced. A 50 kDa protein was detected in CTV-infected tissue extract using the RdRp specific antiserum. However, the detection of this protein was difficult and required the use of highly sensitive detection

methods. The detection of a protein with antiserum specific to the recombinant RdRp suggests that the RdRp gene of CTV is expressed in the infected tissue, and implies that the proposed +1 translational frameshift takes place in the CTV infected tissue. The size of the detected protein is smaller than the expected size of the CTV RdRp. This indicates that after the expression of RdRp as a fusion protein with the CTV polyprotein, it is processed and cleaved from the rest of the polyprotein encoded by CTV ORF1a. The 50 kDa protein was detected in the cytoplasmic and membrane enriched fractions of infected tissue, and mostly accumulated in the membrane fraction and not in healthy tissue extracts. This is consistent with the sub-cellular localization of RdRp and replication-associated proteins of other RNA viruses.

CHAPTER 5
CHARACTERIZATION OF THE +1 TRANSLATIONAL FRAMESHIFT FOR THE RNA-DEPENDENT RNA POLYMERASE GENE OF CTV

Introduction

Ribosomal frameshifting is a directed change in the translational reading frame, which allows the production of a single protein from two overlapping genes. The frameshift can occur in either the 5' (-1 frameshift) or 3' (+1 frameshift) direction (Brierley, 1995). A number of viruses and mobile genetic elements use the ribosomal frameshifting mechanism to control expression of their replicase gene at the translational level. This mechanism facilitates controlled low-level synthesis of the polymerase, which is needed in only small quantities. (Brault and Miller, 1992). The -1 programmed ribosomal frameshifting has been demonstrated for animal retroviruses, coronaviruses, toroviruses, arteriviruses and astroviruses (Brierley, 1995; Farabaugh, 1996), a yeast double-stranded RNA virus (Dinman and Wicker, 1994) and also for the plant luteoviruses, sobemoviruses, carlaviruses, enamoviruses and dianthoviruses (Maia et al. 1996).

Sequence motifs responsible for inducing the -1 ribosomal frameshift were identified by extensive sequence comparison and site-directed mutagenesis of the frameshift region of these viruses. The -1 frameshift signal consists of two cis-acting sequence elements including a shifty heptamer with the consensus sequence of X XXY YYZ (the triplets represent 0 frame, X=A, G or U; Y=A or U; Z=A, C or Y), called the “slippery” sequence and a down stream RNA secondary structure in the form of a stem-loop or a more complex configuration, the pseudoknot (Brierley, 1995; Farabaugh, 1996).

On the other hand, +1 ribosomal frameshifting is less common, and it has been described in only a few systems including the yeast retrotransposon TY (Clare and Farabaugh, 1985; Clare et al. 1988), the copia-like element of Drosophila (Farabaugh, 1997) *Leishmania* dsRNA virus (Lee et al. 1996) and cellular genes such as rat ornithine decarboxlyase antienzyme gene (Matsufuji et al. 1995) and peptide release factor 2 (prfB) gene of *E. coli* (Craigen and Caskey, 1986). The +1 frameshifting has been proposed for plant closteroviruses (Agronowsky et al. 1994), including citrus tristeza virus (CTV) (Karasev et al. 1995).

The +1 frameshift was first reported for the yeast retrotransposon Ty-1 in which TYA ORF coding for the gag analog overlaps with the first 38 nucleotide of the TYB ORF coding for the pol analog of the retroviruses. The +1 translational frameshift occurred within the conserved sequences of the overlap between TYA and TYB ORFs (Clare and Farabaugh, 1985). Based on the analyses of the 38 bp overlap region, it was first suggested that 31 nt in that region were required for the frameshift (Wilson et al 1986) and later the length of the required sequences was reduced to 14 nt (Clare et al. 1988). Further analysis of this sequence demonstrated that only 7 nt (CUU-AGG- C) were necessary and sufficient to promote the +1 frameshift in Ty-1 (Belcourt and Farabough,1990). The heptomeric minimal signal required for the +1 frameshift contained two overlapping leucine codons (CUU and UUA) and an arginine codon (AGG) decoded by a low-abundance tRNA which is encoded by a single copy gene, called rare arginine. Based on this information, the tRNA slippage model was proposed for the mechanism of +1 frameshift in which the ribosome pauses at the rare arginine codon, tRNA slips from the cognate to the near cognate codon and the frameshift takes place with translation continuing in the +1 frame (Farabough,1996; 1997). This model was supported by the report that over expression of arginine CCU tRNA suppressed the +1 frameshift in yeast

(Kawakami et al. 1993). More recently it was reported that the pokeweed antiviral protein also specifically inhibited the Ty-1 directed +1 ribosomal frameshift in yeast (Tumer et al. 1998).

Genomic sequence analysis of CTV revealed that ORF 1a and 1b overlap by 123 nucleotides indicating that the RNA-dependent RNA polymerase (RdRp) of CTV encoded by the ORF 1b is possibly expressed via a +1 frameshift in the 5' end of ORF1a (Karasev et al. 1995). The overlap region had no homology to the characterized +1 frameshift signals of other systems but showed some similarity with the overlap region of beet yellows virus (BYV), the type member of the closterovirus grup. Although the overlap region of ORF1a and 1b is relatively conserved, there were significant differences between the BYV and CTV sequences around the predicted frameshift site. The overlap region of BYV contained a UGA stop codon, a GGGUUU slippery sequence and a stem-loop structure (Agranovsky et al. 1994) which were similar to -1 frameshift signals and absent in the CTV sequence (Karasev et al. 1995). The absence of these elements in CTV indicated that the mechanism of the +1 frameshift used by BYV and CTV, two closely related viruses, may differ. An arginine codon CGG in the overlap region was one of the least frequent codons used in the CTV genome, and it aligned with the UAG stop codon in the overlap region of BYV. Based on this information, it was suggested that this rare arginine codon may be important for the +1 frameshift of CTV, and the mechanism of the frameshift may be similar to that of Ty-1. The rare arginine codon may serve as a stop codon to pause the ribosome during translation and shift the reading frame in +1 direction (Karasev et al. 1995). These proposals have never been tested experimentally and the occurrence of the frameshift has not been demonstrated for CTV, BYV and other

closteroviruses *in vivo* or *in vitro*, and the mechanism of the frameshift still remains to be determined.

In this study, expression of the RdRp gene by the +1 frameshift was demonstrated using an *in vitro* translation system and an *Agrobacterium*-mediated transient expression assay. The involvement of the overlapping region of ORF 1a and 1b and the possible sequence elements required in the +1 frameshift were analyzed by the *Agrobacterium*-mediated transient expression assay using the $-glucronidase (GUS) and the green fluorescent protein (GFP) as fusion reporter genes.

Material and Methods

In vitro Transcription and Translation

To determine if the proposed +1 frameshift for the expression of the RdRp actually occurs in CTV, the 3' half of the ORF1a containing the helicase domain and the complete ORF1b was amplified from a clone containing both regions of the CTV sequences by PCR using primers CN437 and CN257 (Table 5-1) (Figure 5-1). An ATG translation initiation codon and *Nde*I restriction site at the 5' end and *Bgl* II restriction site at the 3' end were incorporated by primers CN437 and CN357, respectively, during PCR amplification. The amplified DNA fragment was cloned between *Nde* I and *Bgl* II sites in the pGEM-T vector under the SP6 promoter. The clone was sequenced, and the presence and the integrity of the sequences for the helicase, the overlap region of ORF1a and ORF1b and the whole ORF1b were confirmed. The CTV sequence in the clone was transcribed and translated using the TNT *in vitro* transcription and translation kit (Promega) with wheat germ extract and ^{3}H labeled leucine according to manufacturer's instructions. The translation products were separated by 12 % SDS-PAGE and detected by autoradiography.

Table 5-1. The sequences of the primers used for amplification, mutagenesis and cloning of the frameshift constructs.

Primer	Sequence[a](5' to 3')	Orientation
CN257	cta*ctcgaga*tctATCAATCGATCAGCCGGTT	Anti-sense
CN356	aaa*gcggccgc*a*ccatgG*AGACACTGCCCCTCCCGACTCC	Sense
CN365	atga*ccatgG*AATATAAGGGTAGTAAAGC	Anti-sense
CN367	ctttactagtCGACTCTCGTACGACAGAAGG	Anti-sense
CN368	ctttactagtaat**a**TTACGACTCTCGTACGACAGAAGG	Anti-sense
CN416	ctttactagtaaatattACGACTCTCGTACGACAGAAGG	Anti-sense
CN417	ctttt*actagtaatatt*ATATGGTAACATTATCACACCC	Anti-sense
CN424	GC**A**C**GC**GTCCGG**A**G**ATC**TAA**A**GT**TA**CA**A**GCAATTCCTCCAA	Sense
CN425	**T**TTA**GAT**C**T**CCGGAC**GC**G**T**GCAATTTCATGTAAGTTACCGG	Anti-sense
CN426	**AAACCATGGTAGATCTGA**CGG**TGTGAGCAA**GGGCGAGGAG	Sense
CN427	TCGCCC**TT**G**CTCACACTA**GCC**ACAGATCTACCATGGTTT**	Anti-sense
CN428	GGCTCGTGTT**A**GGCGTAGTAAGG	Sense
CN429	CCTTACTAC**T**CCTAACACGAGCC	Anti-sense
CN437	agagctcatatgGTGTCCTATAGGTGTCCTTG	Sense

[a]Lower case letters in the 5' ends of the primers indicate the non-viral sequences such as restriction sites and initiation codon and extra sequences incorporated at the ends of the CTV sequences. The bold upercase letters show the specific mutation introduced in to the overlap region.

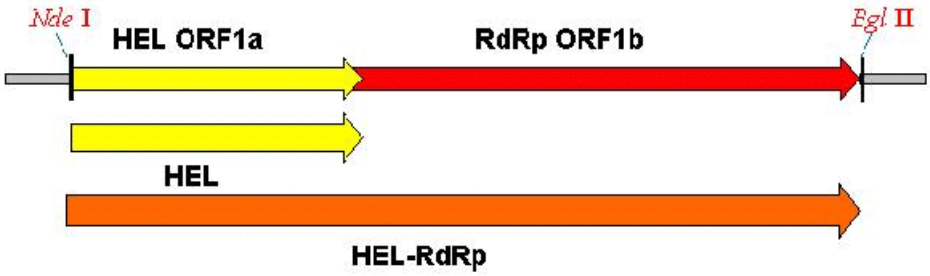

Figure 5-1. The cloning of the CTV ORF1a and 1b for *in vitro* transcription and translation. The CTV sequence was cloned between NdeI and BglII sites in the pGEM-T plasmid under the SP6 promoter. The ORFs and expected translation products are shown by the different bars.

Amplification, Mutagenesis and Cloning of Overlap Region of CTV

Three fragments, called FS-1, -2 and -3 were amplified using primer pairs CN365-367, CN365-416 and CN365-368, respectively (Figure 5-2). An ATG translation initiation codon and *Nco*I restriction site at the 5' end, and a Spe I restriction site at the 3' end were incorporated into all three fragments during PCR. In addition, the native stop codon of CTV ORF1a was removed from the 3' end of FS-1, and one and two extra nucleotides (T and TT) were introduced after the stop codon in 3' end FS-3 and FS-2, respectively during PCR amplification. The amplified fragments FS-1, -2 and -3 were cloned into pCambia1303 (pC1303) plasmid between the cauliflower mosaic virus (CaMV) 35S promoter and the reporter GUS-GFP gene at the *Nco*I and *Spe*I sites so that the GUS-GFP gene was in the 0, -1 and +1 frames in relation to the first ATG codon, respectively. The resulting plasmids were designated as pC1303FS-1, pC1303FS-2 and pC1303FS- 3 (Figure 5-2 and 5-3). In the pC1303FS-1 plasmid, the GUS/GFP gene was fused to the CTV sequences without the stop codon in 0 frame to test the expression of GFP in the ORF1a. This construct was used as the control for the expression of the GUS-GFP. In the pC1303FS-2, the GUS-GFP gene was fused to the CTV sequence (with the stop codon and added TT) in the -1 frame to test if the cloned sequence could function as a -1 frameshift signal and induce expression of the GUS-GFP cloned in the -1 frame. In the pC1303FS-3, the GUS-GFP gene was fused to the CTV sequence (with the stop codon and one added T) at the +1 frame to test if the sequence of the cloned region contained the frameshift signal and could induce expression of GUS-GFP in the +1 frame (Figure 5-2 and 5-3).

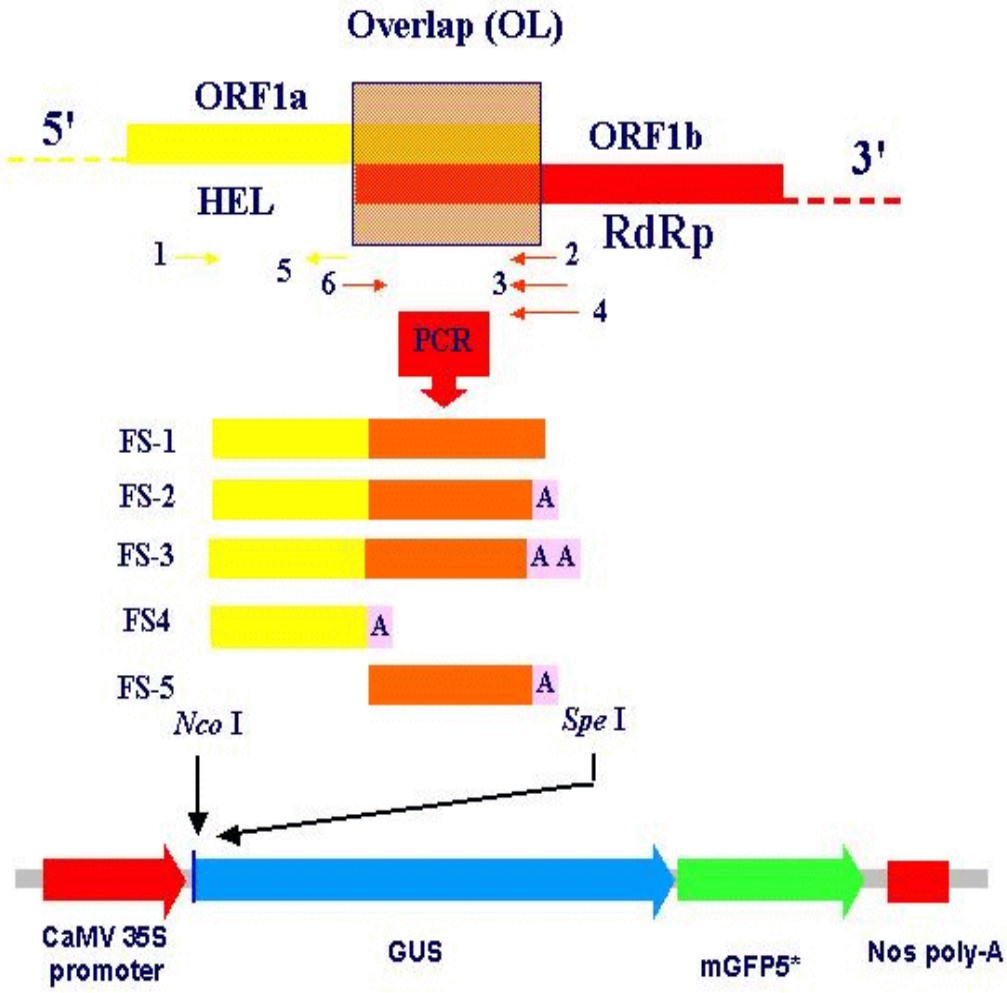

Figure 5-2. Amplification mutagenesis and cloning strategy used for the constructs of overlap region of CTV ORF1a and 1b. The arrows with the numbers 1, 2, 3, 4, 5, and 6 indicates the primers CN365, CN367, CN368, CN416, CN417 and CN356, respectively. The sequences of these primers are shown in Table 5-1.

Since the constructs pC1303FS-1, -2 and -3 contained sequences in addition to the 123 nt overlap region, it also was necessary to make individual constructs containing only the 123 nt overlap region and the upstream helicase sequence. Therefore, the upstream and the overlap sequences were individually amplified by PCR using primer pairs CN365-CN417, and CN356-CN368 (Table 5-1) and designated as FS-4 and FS-5, respectively. An ATG translation initiation codon and *Nco* I restriction site at the 5' end, and an Spe I restriction site and one extra nucleotide T at the 3' end were incorporated into both FS-4 and FS-5 during PCR amplification. These fragments were cloned into the pC1303 between the CaMV 35S promoter and the reporter gene at the *Nco* I and *Spe* I sites to make the GUS-GFP reporter gene in the +1 frame in relation to the first ATG codon, and the resultant plasmids were called pC1303FS-4 and pC1303FS- 5, respectively (Figure 5-2 and 5-3).

To study the importance of the conserved secondary structure identified in the overlap region and the significance of the rare arginine codon proposed to be involved in the +1 frameshift, more constructs with specific mutations were generated by the overlap extension PCR method (Ho et al. 1989). First, sense and anti-sense internal oligonucleotide primers (CN424-5, CN426-7 and CN428-9) with the desired mutation were designed for each construct. They were used with an external primer (CN356 or CN368) specific to the 3' and 5' ends of the overlap sequence for PCR amplification of two overlapping DNA fragments. Then, these DNA fragments were mixed and used as template for a second round of PCR amplification using the external primers (CN356 and CN368) to produce the complete (123 nt) overlap sequence with the desired mutations. Three mutant overlap sequences, FS-6, FS-7 and FS-8, were generated using this method. They were cloned into the pC1303 plasmid

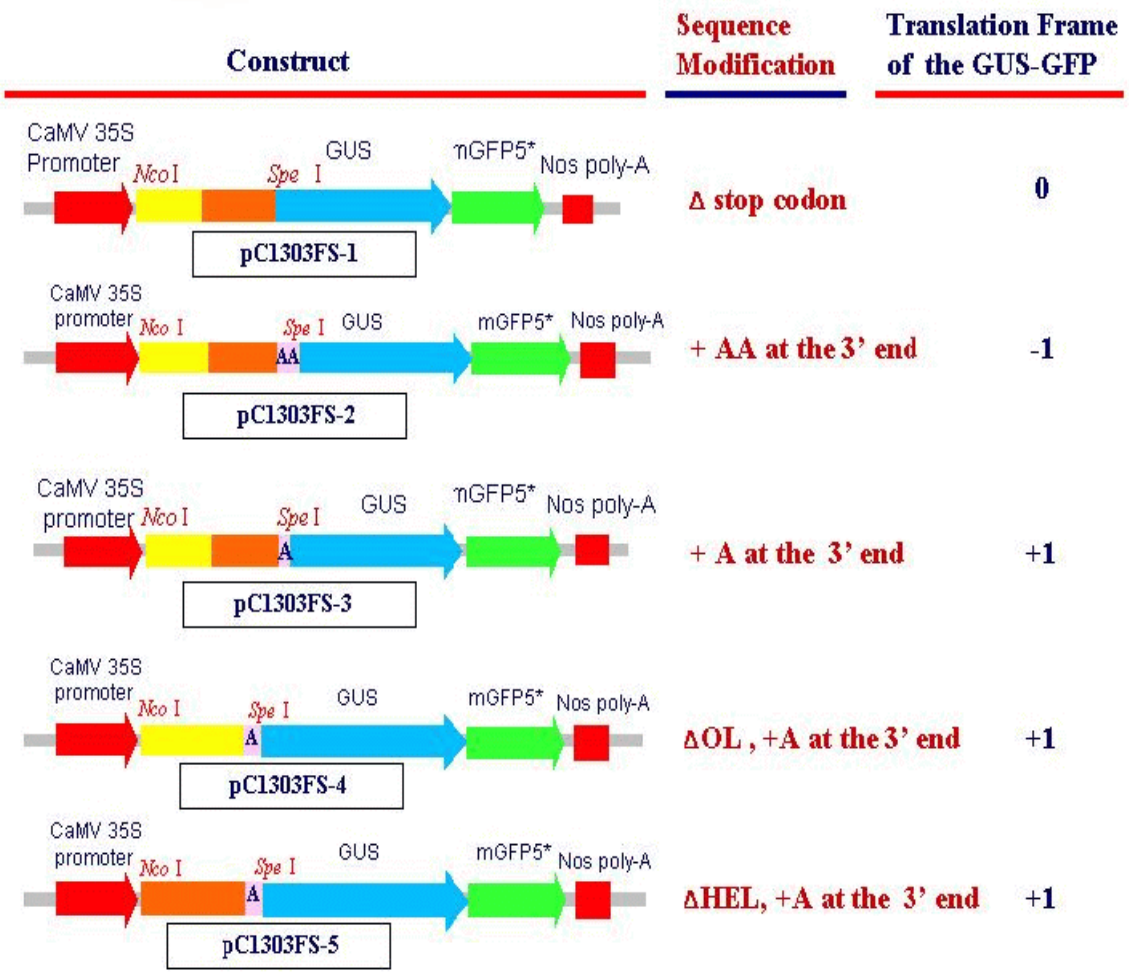

Figure 5-3. The constructs prepared for the overlap region of CTV ORF1a and 1b in pC1303 plasmid vector. The orange boxes indicate the 123 nt overlap sequences, and the yellow boxes indicate the helicase sequences just upstream of the overlap region. The sequence modifications and the translational frame for the reporter genes in relation to the first ATG initiation codon are shown for each construct.

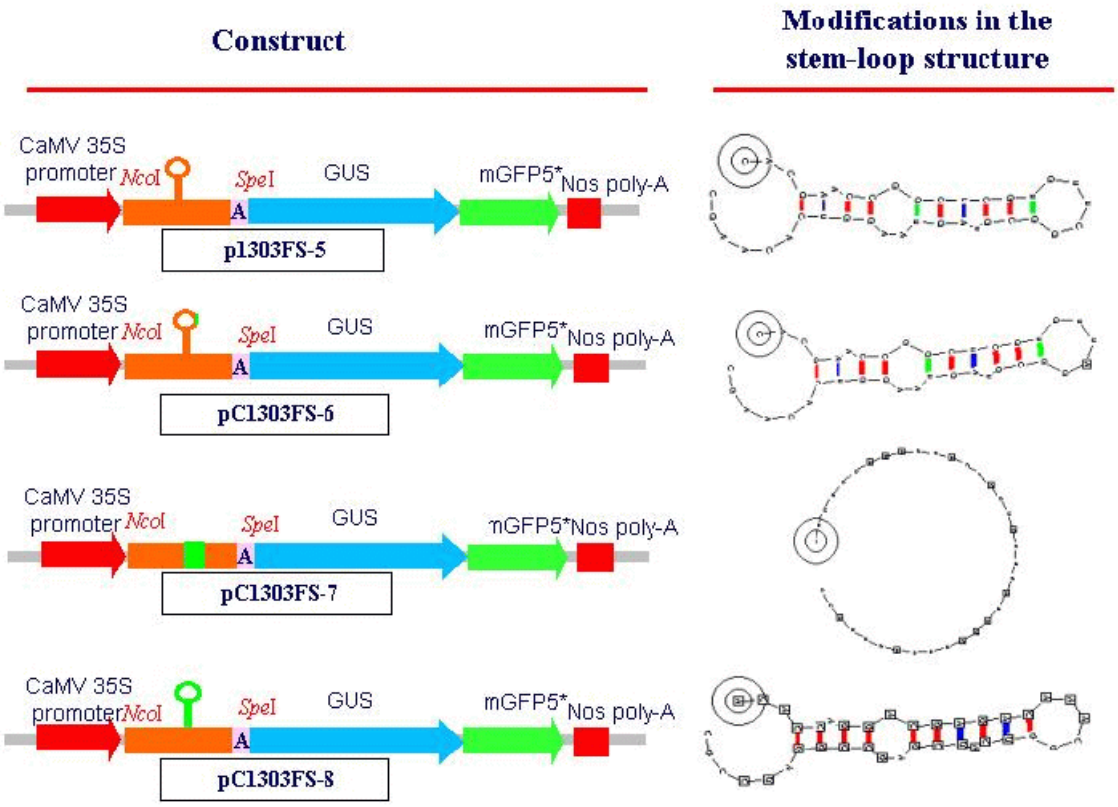

Figure 5-4. The pC1303 plasmid vector containing the constructs of the overlap region of CTV ORF1a and 1b with mutations in the predicted stem-loop structure and the proposed rare arginine codon. The orange boxes indicate the 123 nt overlap sequences, and the mutations in this region are shown by green color in the construct and by boxes in the structures on the left.

as were the previous constructs in which the GUS-GFP reporter gene was in +1 frame in relation to the first ATG codon. The resulting plasmids were designated as pC1303FS-6, pC1303FS-7 and pC1303FS-8, respectively. In the pC1303FS-6 plasmid, the rare arginine codon CGG was changed to AGG the most frequent arginine codon in CTV, by a single mutation. In the pC1303FS-7, the stem-loop structure was resolved by changing 12 of the 37 nt involved in the structure which resulted in only a single change in the amino acid sequence. The pC1303FS-8 contained 30 mutations in the the 37 nt secondary structure, but this completely different and unrelated sequence still formed an identical stem-loop structure in the same region (Figure 5-4). All plasmids were maintained in *E. coli* and later introduced into *Agrobacterium* strain AGL1 by triparental mating or transformation of *Agrobacterium* with cold shock.

Sequence Analysis of the Overlap Region of Different CTV Isolates

The sequences of the overlapping region between the ORF1a and 1b from the CTV isolates T30, T36, T66, B53, B165, B185, B249, SY568, T385,VT, and 3800 were obtained as described in the Chapter 3. The sequences were assembled, aligned and analyzed using GCG, and Vector NTI suite. The secondary structures for the sequences were detected and analyzed using the RNA Draw program.

Transient Expression Assay

A previously reported *Agrobacterium*-mediated transient gene expression system (Kapila et al 1997) was modified and used to study the effect of CTV sequences in the expression of the GUS-GFP reporter genes (Figure 5-5).

Plant Materials

Initially, fully expanded leaves taken from 4-6 week old *Nicotiana benthamiana* and 2-3 months old *Citrus sinensis* var. Madam Vinus seedlings maintained at 25 C, were used for infiltration. Later, only leaves from two months old *in vitro* grown *Citrus paradisi* var. Duncan was used in all infiltration experiments.

Preparation of the *Agrobacterium* Suspension

Agrobacterium strain LBA4404 or AGL1 containing the pC1303 control plasmid, and pC1303FS-1 to -8 frameshift test constructs were inoculated into YEP medium (10 g/l Bactopeptone, 10 g/l yeast extract and 5 g/l NaCl pH 7.0) containing the appropriate antibiotics. They were grown overnight to log phase (OD_{600nm}= 0.7-0.8) at 280 rpm and 28 C. The cultures were centrifuged at 4 ^{0}C and 5000 rpm for 5 min, and the pellets were re-suspended to a final concentration of $5x10^8$ cfu/ml with MS medium containing 4.3 g/l MS salt, 100 mg/l myo-inoistol, 30 g/l sucrose and 150 μM acetosyringone.

Infiltrations of the Leaves with *Agrobacterium* Suspensions

The leaves were detached from citrus seedlings or tobacco plants and placed onto wet and sterile Whatman filter papers fitted into petri plates. One milliliter of the *Agrobacterium* suspension was infiltrated into each leaf using a 1-ml syringe without a needle (Figure 5-5). Plates were sealed and incubated in a growth room at 25 ^{0}C and a 16 hr light/8 hr dark photo-period for 4-5 days.

Fluorescent Microscopy

The expression of GFP in the citrus and tobacco leaves infiltrated with *Agrobacterium* was first analyzed using a dissecting microscope (Zeiss) with a fluorescent light source with

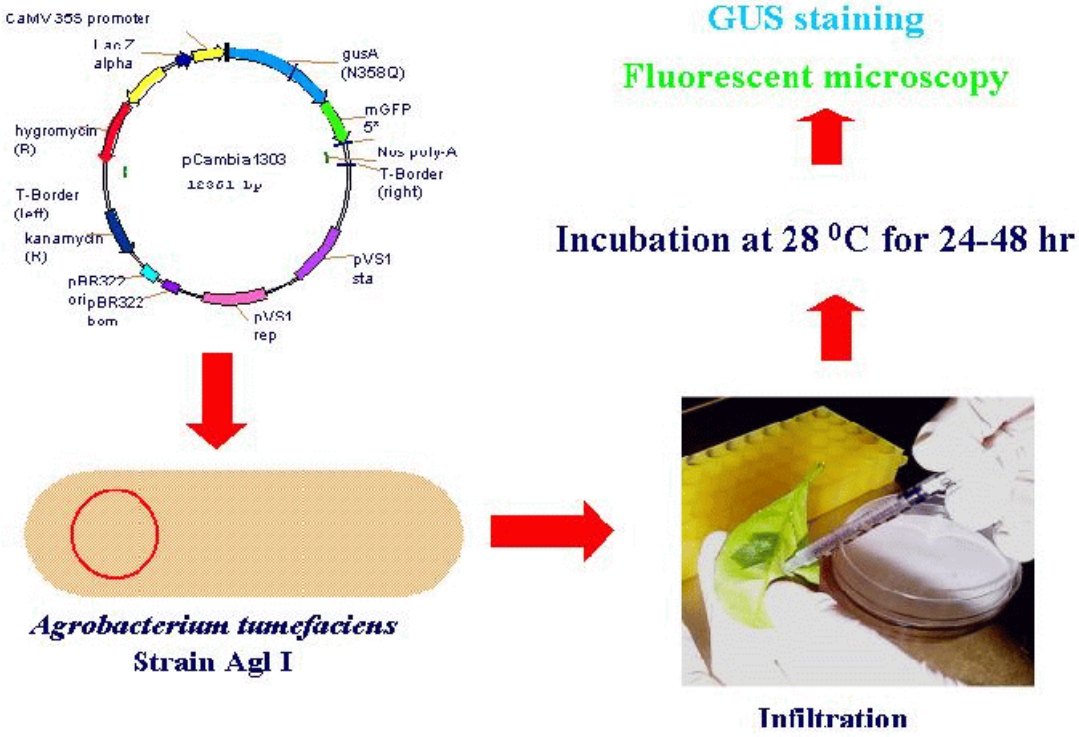

Figure 5-5. The flowchart of the *Agrobacterium*-mediated transient expression assay used to study the involvement of the overlap region of CTVORF1a and 1b in the putative CTV + 1 frameshift.

a 515 nm long pass emission filter transmitting red and green light and a 450-490 nm excitation filter. The fluorescent images were photographed using a 35 mm camera attached to the fluorescent microscope.

Histochemical GUS Staining

The leaves were placed in small petri dishes or 2-ml eppendorf tubes containing 50 mM $NaPO_4$ pH 7.0 10 mM Na_2EDTA solutions and 5 mg/ml 5-bromo-4-chloro-3-indolyl- -D-glucuronide (X-Gluc). A mild vaccum was applied for 5 min to distribute the substrate equally in the cells of the leaves. The leaves were incubated in the solution overnight at 37 ^{0}C, and then they were cleared using a solution of 70% ethanol and 30% acetic acid. The reactions were photographed with a digital camera.

Results and Discussion

In vitro Transcription and Translation

Coupled *in vitro* transcription and translation of the pGEMT-HEL-RdRp construct produced two expected bands with estimated molecular weight of 82 and 30 kDa, but also a 47 kDa unexpected band (Figure 5-6). No translation product was produced with the pGEM-T plasmid alone (data not shown). The size of the 30 kDa protein corresponds to the protein product of the helicase domain, and the size 82 kDa protein corresponds to the fusion protein product of both helicase (ORF1a) and the RdRp (ORF1b) of CTV. Since the translation without frameshift could only produce the 30 kDa protein, and the 82 kDa protein could not be produced unless the frameshift occurred, the detection of the 82 kDa band demonstrates that the +1 frameshift took place during the *in vitro* translation. This result clearly shows that the RdRp gene of CTV is in fact expressed by the +1 frameshift. The amount of the 30 kDa protein was significantly higher then the 82 kDa protein because the frameshift usually occurs

infrequently. Quantification of these proteins showed that the amount of the 82 kDa protein was about 1-5% of the amount of 30 kDa protein, indicating the efficiency of CTV frameshift was about 1-5%. This result is in agreement with the translation efficiency reported in other systems (Brierley, 1995; Farabaugh, 1996), implying that CTV has a relatively efficient + 1 translational frameshift. The unexpected 47 kDa band detected in the *in vitro* translation system may be the product of alternative translations of the clone, or it might be result of the processing of the 82 kDa protein by the proteases in the wheat germ system.

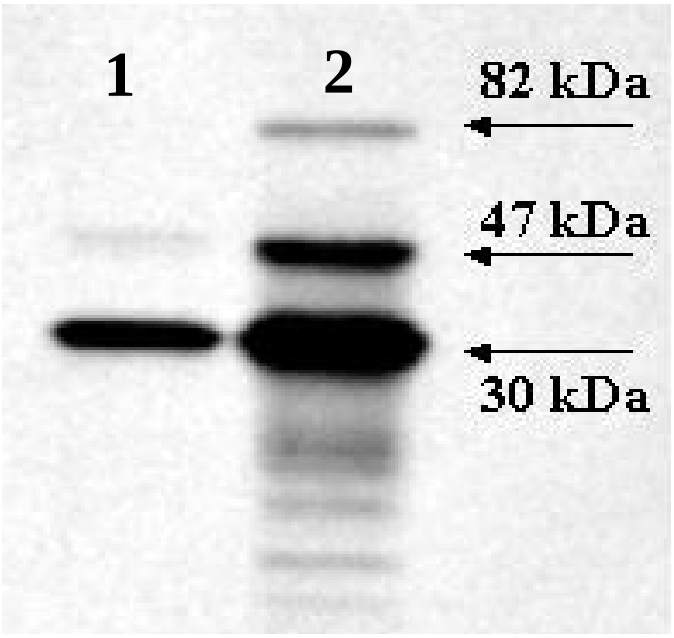

Figure 5-6. *In vitro* transcription and translation of HEL-RdRp construct in wheat germ extract demonstrating the +1 frameshift for the expression of the CTV RdRp gene. Lanes 1 and 2 contain 2% and 10% of the total protein produced by the *in vitro* translation of the HEL-RdRp construct.

Development of Transient Expression Assay

Four citrus and tobacco leaves were first infiltrated with *Agrobacterium tumefaciens* strain LBA4404 or AGL1 with and without plasmid pC1303 containing the GUS/GFP fusion

gene in the inoculation medium. The expression of the GUS and GFP in the infiltrated leaves was monitored daily for four days by histochemical staining and using a fluorescence microscope . Bright green fluorescent spots and blue histochemical staining were observed, both in citrus and tobacco leaves infiltrated with plasmid pC1303, indicating that both GFP and GUS were transiently expressed in the inoculated leaves. The expression of both the GUS and GFP were first detected one day after inoculation (DAI), and maximum expression of GFP was observed two DAI. The expression of both GUS and GFP could still be detected three DAI , but a significant reduction in the level of expression was observed three and four DAI. No GFP expression was detected in uninoculated leaves or those infiltrated with inoculation medium only or LBA4404 or AGL1 without the plasmid. The detection of the both GUS and GFP only in the pC1303-inoculated leaves indicates that green spots were not auto-fluorescence or artifacts due to wounding of the leaves during the infiltration. These results show that an *Agrobacterium*-mediated transient expression assay can be used as an efficient system to analyze involvement of the overlapping region of CTV in the + 1 frameshift. This system can be used also to test plant transformation constructs and to study the function or the effect of other genes and/or regulatory elements in citrus. No significant differences were observed between citrus and tobacco leaves or between *Agrobacterium* strains LBA4404 and AGL1. Therefore, the *Agrobacterium* strain AGL1 and leaves from *in vitro* grown grapefruit were used in following transient assays because they were readily available and commonly used in our laboratory.

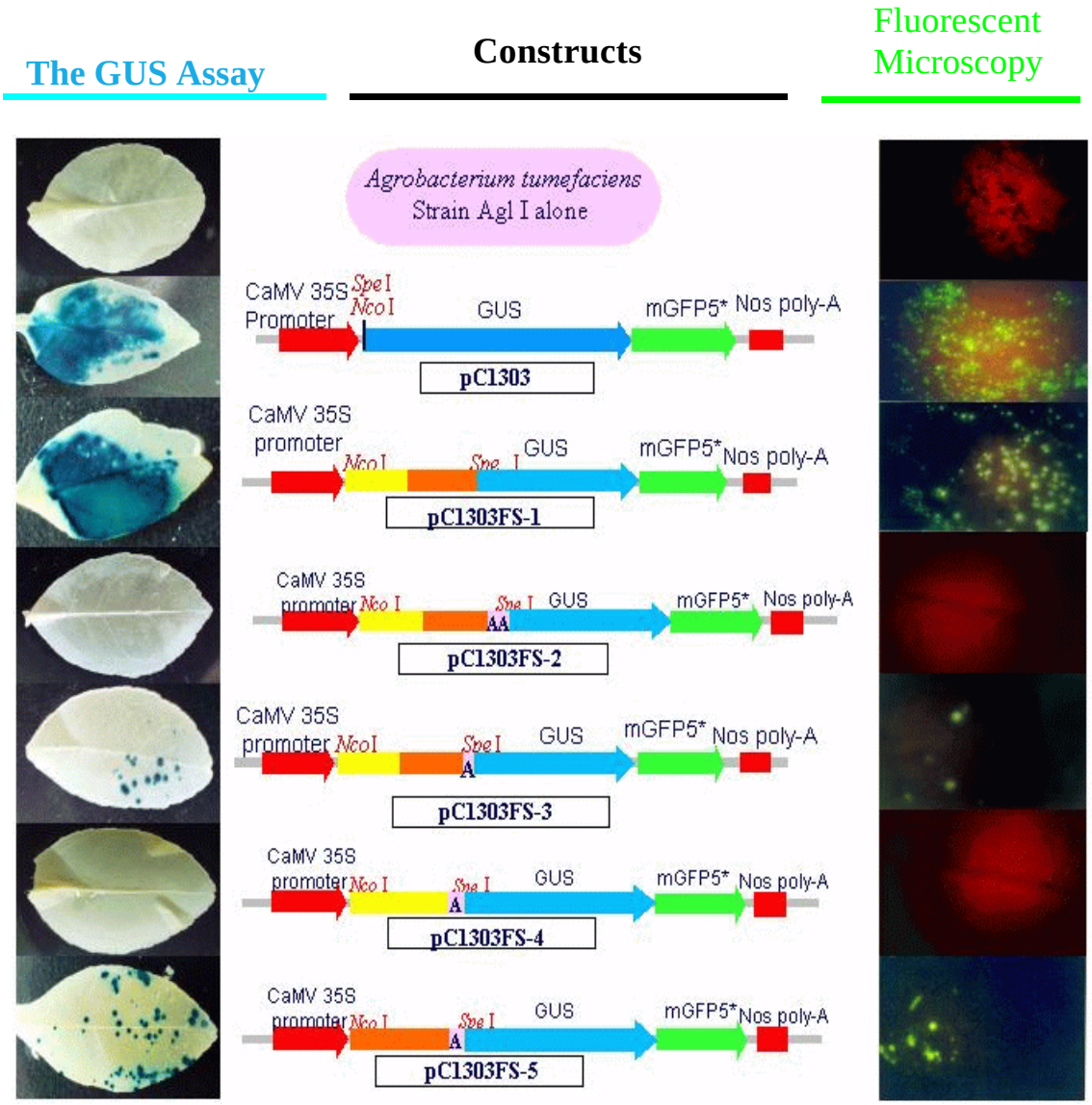

Figure 5-7. Expression of the GUS and GFP reporter genes in citrus leaves infiltrated with the pC1303 containing different constructs of the overlap region of the CTV ORF1a and 1b.

Involvement of the Overlap Region of ORF1a and 1b in the +1 Frameshift

Citrus leaves were infiltrated with individual suspensions of *Agrobacterium* strain AGL1 harboring the control and frameshift plasmids pC1303, pC1303FS-1, pC1303FS-2 and pC1303FS-3. The expression of the GUS and GFP was detected in citrus leaves inoculated with pC1303 and pC1303FS-1 as well as with pC1303FS-3, the +1 frameshift tester plasmid. However, no GUS or GFP expression was detected in uninoculated leaves or leaves infiltrated with inoculation medium or AGL1 without plasmid or with pC1303FS-2, the -1 frameshift tester plasmid (Figure 5-6). In the pC1303FS-3 construct, the GUS-GFP gene was fused to the overlapping sequence at +1 frame in relation to the ATG translation initiation codon (Figure 5-5), and the GUS and GFP could not be expressed unless the +1 frameshift took place. Therefore, the expression of the GUS and the GFP in citrus leaves infiltrated with pC1303FS-3 plasmid demonstrates that the +1 frameshift was induced by the CTV sequence from the overlap and its upstream region. Since the pC1303FS-2 plasmid containing essentially the same sequence did not show any expression of the GUS or GFP in - 1 frame, it was shown that the CTV sequence did not induce the -1 frameshift, and the signals contained in the sequence were specific to the +1 ribosomal frameshifting (Figure 5-7). High levels of GUS and GFP expression were observed in citrus leaves infiltrated with the pC1303FS-1. This was the 0 frame test construct in which the GUS/GFP gene was fused to CTV sequences without the stop codon; thus, expression was expected regardless of the +1 frameshift (Figure 5-7). Even though a variation was observed in the number of cells expressing the GUS and GFP with this construct and the control plasmid pC1303, the level of expression in the individual cells was similar indicating that reporter gene expression was not dramatically affected by the insertion of the CTV sequences. Since frameshifting generally occurs at low

frequency, the expression the GUS and GFP in the leaves infiltrated with the pC1303FS-3 plasmid was expected to be lower than in leaves infiltrated with the pC1303 and pC1303FS-1 control plasmids (Figure 5-7). Although the number of blue stained and green fluorescence spots were significantly lower in the pC1303FS-3 than the control plasmids, the expression of the reporter genes could not be analyzed quantitatively because their expression levels depends on and is affected by variables such as efficiency of infiltration and transformation. Nevertheless, the assay is very simple and efficient for qualitative analysis of whether of not the +1 frameshift occurs with specific constructs.

Since the constructs pC1303FS-1, 2 and 3 contained about 280 nt of CTV sequence, including the 123 nt overlap region and about 160 nt upstream sequences, it was not demonstrated whether the 123 nt overlap sequence by itself is able to induce the +1 frameshift. To determine that, citrus leaves were infiltrated with *Agrobacterium* carrying pC1303FS-4 and pC1303FS-5 as well as control plasmids pC1303 and pC1303FS-1. The expression of the GUS and GFP was observed in leaves infiltrated with control plasmids pC1303 and pC1303FS-1, and the pC1303FS-5 plasmid containing only the 123 nt overlap sequence of CTV ORF1a and 1b (Figure 5-7). On the other hand, no expression of either reporter gene was detected in leaves infiltrated with the pC1303FS-4 plasmid containing the about 160 nt sequence from the helicase domain just upstream of the overlap region of CTV ORF1a and 1b (Figure 5-7). This result shows that the 123 nt overlap region is necessary and sufficient for the induction of +1 frameshift in the transient assay. This result is consistent with the information in the literature that the signals or sequence motifs required for

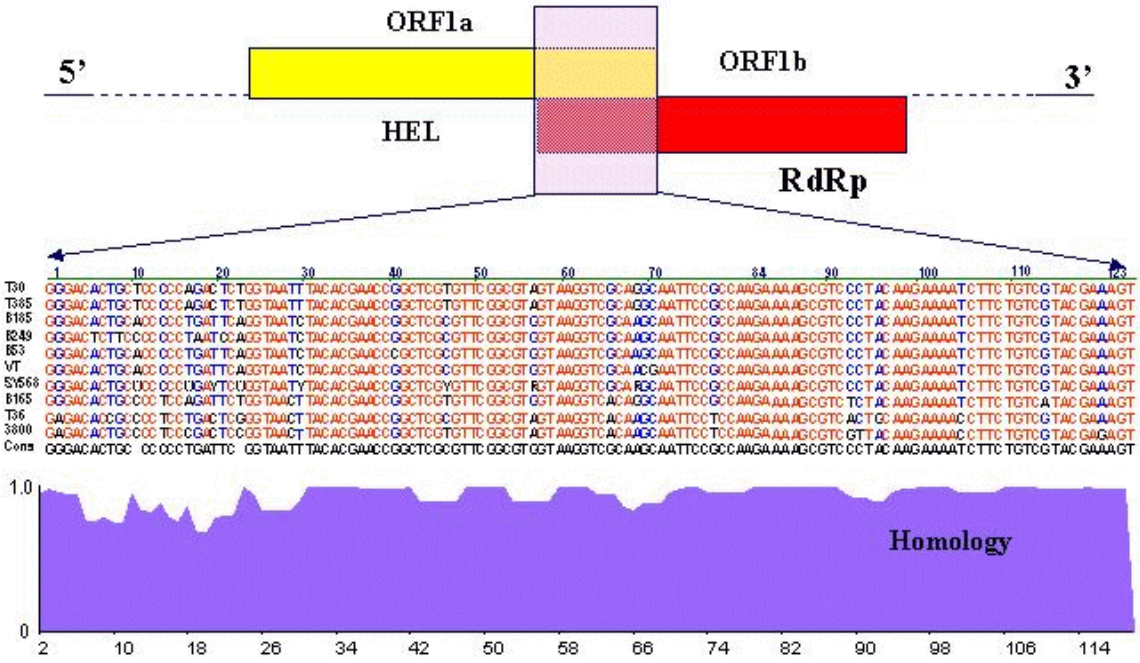

Figure 5-8. Multiple alignment and homology graph of nucleotide sequences of the overlapping region of ORF1a and 1b of 10 biologically and geographically different isolates of CTV. The alignments and the graphs were generated using the Align X program of Vector NTI Suite. The letters W, Y and R indicates T or A, T or C and G or A, respectively. The homology graph displays alignment quality based on the total similarity values (1, 0.5 and 0.2 for identical, similar or weakly similar, respectively) for the residues at a given position..

Table 5-2. The percentage of nucleotide sequence identity of the overlap region of ORF 1a and 1b among ten different isolates of CTV.

Isolates	T30	T36	T385	3800	B53	B165	B185	B249	SY568
T36	88								
T385	100	88							
3800	89	93	89						
B53	92	87	92	86					
B165	93	89	93	90	90				
B185	93	88	93	89	99	93			
B249	90	86	90	84	95	88	95		
SY568	93	86	93	86	92	89	93	90	
VT	91	86	91	85	97	89	91	94	91

frameshifts in most systems are located in the overlap region of two ORFs (Brierley, 1995; Farabaugh, 1996). It also has been reported that signals or motifs required for a +1 frameshift can be composed of a complex secondary structure (Lee et al. 1996) or simple sequences as few as seven nt (Belcourt and Farabough, 1990) located in the overlap region of two ORFs. Further analysis of the overlap region of CTV ORF1a and 1b was necessary to determine if specific sequence elements or motifs of the overlap region were involved in the +1 frameshift and required for the expression of the RdRp gene of CTV.

Sequence Comparison and Secondary Structure Analysis of the Overlap Region

In order to identify possible sequence elements or motifs that may be present in the overlap region and required for the +1 frameshift, sequences of the overlapping region from six different CTV isolates (T30, B53, B165, B185, B249 and 3800) were determined and compared with each other and with the four sequences available in the GenBank, T36, SY568, VT and T385. Multiple alignment and comparison of the sequences indicated that the sequences of overlap regions from biologically and geographically different isolates of CTV are highly conserved (Figure 5-7) and show 84-100% sequence identity among different isolates (Table 5- 3). The most highly conserved sequences were surrounding the rare arginine codon proposed to be involved in pausing the ribosome during the frameshift and at the 3' end of the overlapping region towards the stop codon of the ORF 1a. These regions showed the highest homology and scores (Figure 5-8).

Secondary structures such as pseudoknots and stem-loops have been shown to be involved in frameshifting in other systems. To investigate the presence of secondary structures in the overlap region, the possible folding patterns of the highly conserved overlap sequences were analyzed in the RNA Draw program. The secondary structure analyses of the

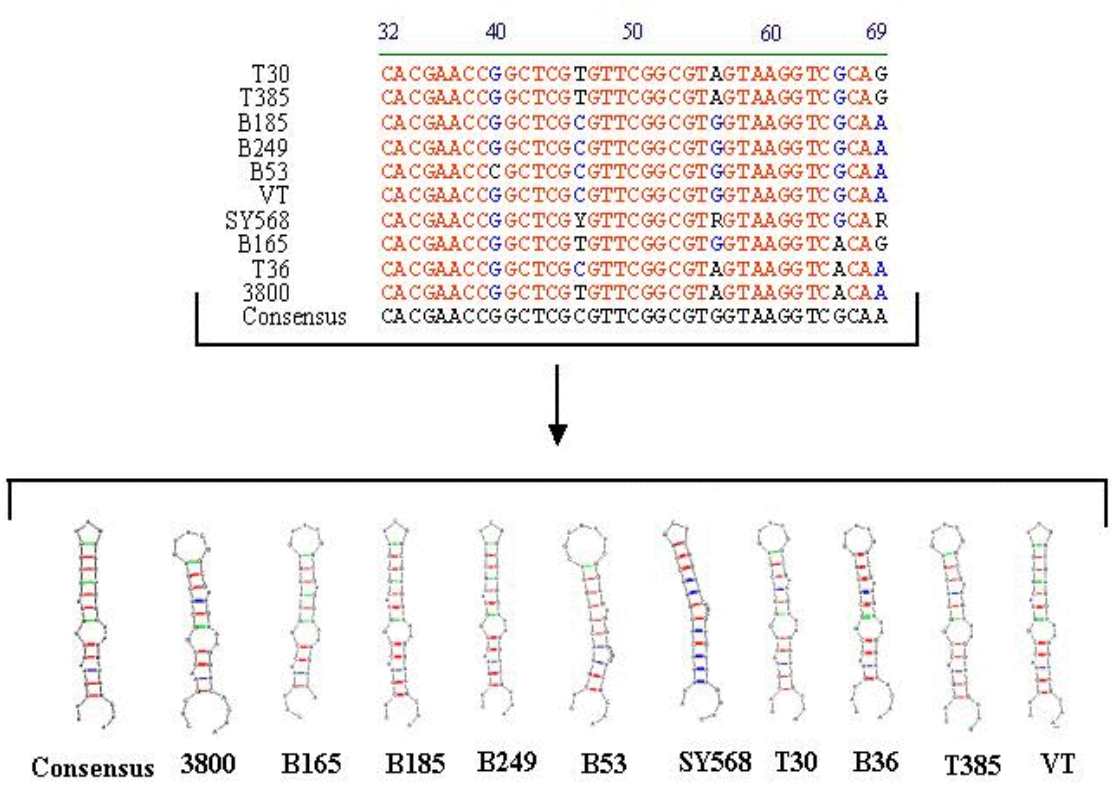

Figure 5-9. Possible folding patterns of the highly conserved nucleotide sequence around the arginine codon in the overlapping region of the ORF 1a and 1b from different isolates of CTV. The secondary structures were generated using the RNA Draw program the letters Y and R indicates T or C and G or A, respectively.

overlap region of isolate 3800 revealed the presence of a stem-loop structure composed of 37 highly conserved nucleotides between positions 9387 and 9423 which is around the arginine codon proposed to be involved in pausing the ribosome during frameshifting. An almost identical stem-loop structure was also found in the same region of the ten biologically and geographically different isolates of CTV (Figure 5-9). Since stem-loop structures were reported to be involved in frameshifting in other viruses, and the structure was conserved in all isolates of CTV, it was possible that the stem-loop structure might also be involved in the +1 frameshift for the expression of the RdRp of CTV. Although the + 1 frameshift characterized in Ty-1 retro transposon does not require any secondary structure, the presence

and requirement of a stem loop structure was reported for *Leishmania* dsRNA virus (Lee et al. 1996). Therefore, it was worthwhile to study the possible role of the conserved secondary structure found in the overlap region of CTV ORF1 an 1b.

The Role of the Secondary Structure and the Rare Arginine Codon in the +1 Frameshift

To study the requirement of the conserved stem-loop structure and the rare arginine codon in the +1 frameshift, citrus leaves were infiltrated with *Agrobacterium* containing plasmids pC1303FS-6, pC1303FS-7 and pC1303FS-8 as well as control plasmid pC1303 and pC1303FS-1. The expression of GUS and GFP were observed in leaves infiltrated with control plasmids pC1303, pC1303FS-1 as well as with plasmids pC1303FS-6 and pC1303FS-8. The detection of the GUS and GFP expression in leaves infiltrated with pC1303FS-6 demonstrated that the mutation in the rare arginine codon (CGG to AGG) did not eliminate the frameshift which indicates that the rare arginine codon by itself is neither the only signal nor the major component of the sequence required for the +1 frameshift in this assay. The expression of the reporter genes in the leaves infiltrated with plasmid pC1303FS-8 containing a modified overlap region with a stem-loop structure composed of a completely different sequence than the native overlap region, but not with pC1303FS-7 containing the overlap sequence without the conserved stem-loop structure demonstrates that the secondary structure is important for the frameshift. The elimination of the +1 frameshift with mutation in 12 of the 37 nt involved in the secondary structure indicates that either the secondary structure itself or the mutated 12 nt sequence or the combination of both the sequences and the stem-loop structure is required for the +1 frameshift. Additional

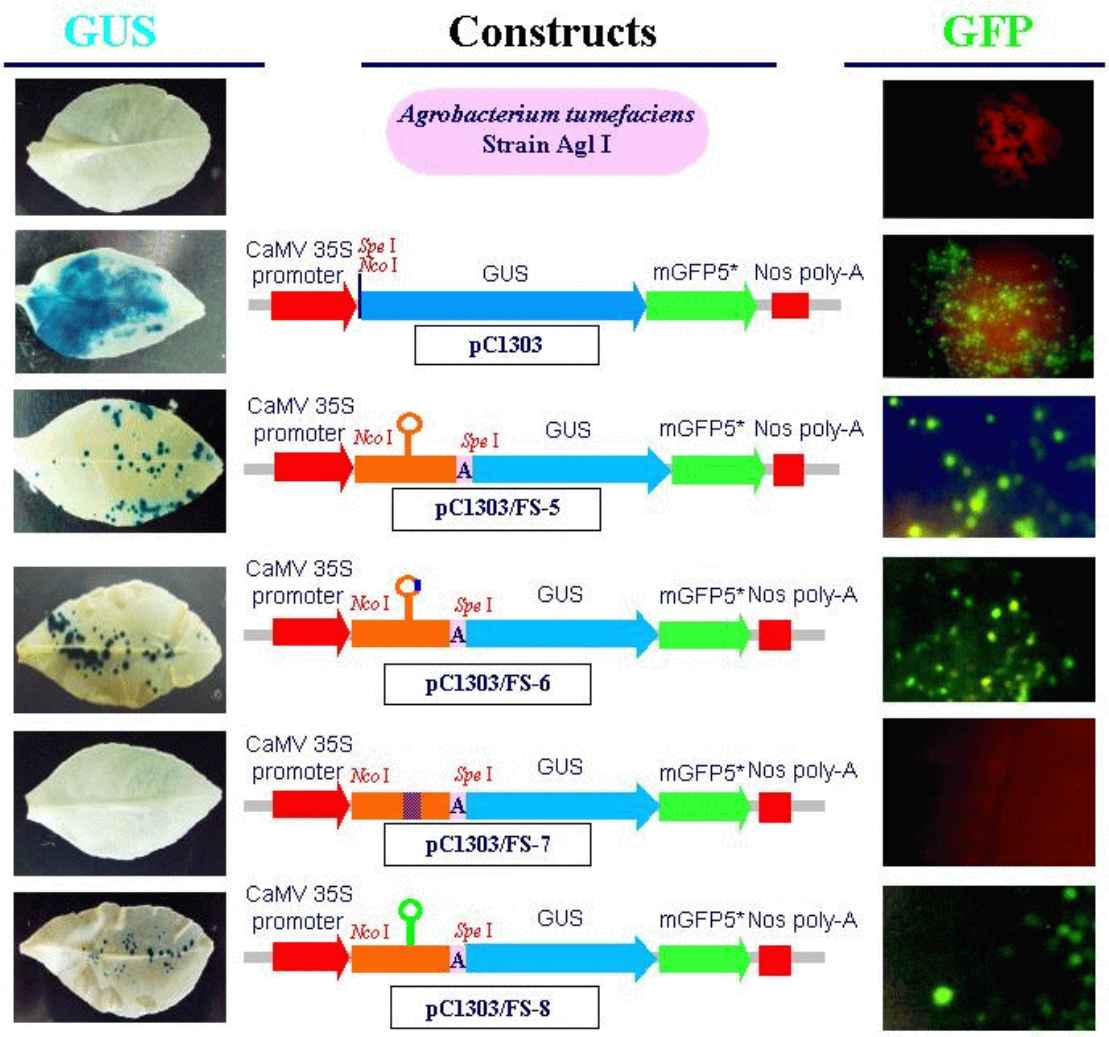

Figure 5-10. Expression of the reporter genes GUS and/or GFP in citrus leaves infiltrated with pC1303 plasmids containing the native and mutant overlap region sequences of ORF1a and 1b. The orange color indicates the native sequences and the mutations are shown by green color over the orange.

constructs with more specific mutations in the stem-loop structure or with specific deletions or more specific mutations in the sequence could provide a better understanding of the role of the overlap sequences in the +1 frameshift.

Conclusions

The requirement of the +1 frameshift for the expression of RdRp gene of CTV was demonstrated using a coupled *in vitro* transcription and translation assay. This assay demonstrated that the efficiency of the +1 transactional frame shift was about 1-5% . An *Agrobacterium*-mediated transient expression assay was adopted and used to analyze the effect of the overlapping region of ORF1a and 1b of CTV in the +1 frameshift. The assay is a rapid and efficient way to analyze CTV sequences fused to the GUS-GFP bi-functional reporter gene in citrus leaves. Using the transient expression assay, it was demonstrated that the overlap region of ORF1a and 1b of CTV promotes the +1 frameshift required for the expression of the CTV RdRp gene. The 123 nt overlap sequence itself is necessary and sufficient to induce the +1 frameshift, as indicated by the expression of both the GUS and GFP reporter gene cloned in the +1 frame position in relation to the ATG translation initiation codon. It was shown that the upstream sequence from the helicase domain was not necessary for the + 1 frameshifting. Involvement of the overlap region of the CTV ORF1a and 1b is consistent with the other frameshifting systems where signals or the sequences required for the frameshifting are located in the overlap region of the two ORF where one of them is expressed by frameshifting.

Sequence analyses of the overlap region of the CTV ORF1a and 1b revealed that the region is highly conserved among geographically and biologically different isolates of CTV. A conserved secondary structure is present in the overlap region of all the isolates of CTV

examined. Sequence modification of the secondary structure eliminated the +1 frameshift, indicating that the stem-loop structure is required for the frameshift. On the other hand, the rare arginine codon was not required and even may not be involved in the frameshift, because the mutation of the rare arginine (CGG) to a more frequent arginine codon (AGG) did not have any effect on the frameshift in the transient assay. Although the individual elements of the frameshift signal remain to be determined, it was clearly shown by both *in vitro* translation and the *Agrobacterium*-mediated transient assay that the RdRp gene of CTV is expressed by the +1 frameshift induced by the overlap sequences of CTV ORF1a and 1b. Since it has been reported that the +1 frameshift of Ty-1 in yeast is specifically inhibited by pokeweed antiviral protein (Tumer et al. 1998), the inhibition of the +1 frameshift with this protein or other molecules or genes provides a specific target for engineering resistance to CTV.

CHAPTER 6
GENETIC TRANSFORMATION OF *Citrus paradisi* WITH THE RNA DEPENDENT RNA POLYMERASE GENE OF CTV

Introduction

The concept of pathogen-derived resistance (PDR) was first proposed as a strategy to produce resistance against a specific or range of pathogens by transforming host cells with genes or sequences derived from the pathogen genome (Sanford and Johnson, 1985). The pathogen-derived resistance was first demonstrated against tobacco mosaic virus (TMV) in transgenic tobacco plants expressing the coat protein (CP) gene of TMV (Powell-Abel et al. 1986). Since then, CP-mediated (CPM) resistance has been successfully developed against a number of economically important viruses in the genera *Potyvirus*, *Cucumovirus*, *Ilarvirus*, *Tobravir*us, *Potexvirus*, *Tobamovirus*, *Tobravirus*, *Carlavirus*, and *Luteovirus* in many crop plants including vegetables, fruits, cereals and forage crops (Baulcombe, 1994; Beachy 1994; Hackland et al. 1994; Pappu et al. 1995; Fuchs and Gonsalves, 1997). Morever, several transgenic plants engineered for virus resistance using CP genes have already been commercialized and many more have been evaluated in the field for release (Fuchs and Gonsalves, 1997). On the other hand, attempts to develop PDR using the CP failed completely for many viruses. This promoted to the use of viral genes other than CP for PDR. Plants were transformed with non-coding sequences from the 5' and 3' untranslated region (UTR) of viral genomes (Nelson et al. 1993; Zaccomer et al. 1993), satellite RNAs (Harrison et al. 1987) and defective interfering RNAs (Kollar et al. 1993), as well as non-structural genes encoding the

protease (Maiti et al. 1993; Vardi et al. 1993), the cell-to-cell movement protein (Malyshenko et al. 1993) and the replicase (Carr and Zaitlin., 1993; Palukaitis and Zaitlin 1997) to produce transgenic plants resistant to viruses. Although the results were somewhat variable in different plant-virus systems, the use of non-structural genes, especially movement protein and replication associated proteins such as RdRp, were shown to be a promising strategy for developing virus resistance in transgenic plants (Beachy 1994; Pappu et al. 1995; Palukaitis and Zaitlin, 1997).

Citrus tristeza virus is one of the most important pathogens of citrus and causes significant economical losses in most citrus producing regions of the world. The pathogen derived resistance has not been explored in citrus due to the limitations in plant transformation methods and the complex genome of CTV. Recent improvement in citrus transformation techniques and characterization of CTV genome now enable application of PDR against CTV in citrus. The CP gene of CTV has already been introduced into sour orange (Gutierrez et al. 1997) and Mexican lime (Dominguez et al. 2000) and grapefruit (Febres et al. 2000) to test CPM resistance against CTV. In addition, transgenic Duncan grapefruit plants expressing CP and other sequences from CTV genome were produced (Febres et al. 2000). More recently, transgenic Mexican lime plants expressing translatable and untranslatable forms of the p23 gene of CTV were produced (Ghorbel et al. 2001). It was reported that transgenic plants expressing a translatable p23 gene are showing symptoms similar to CTV-induced symptoms in infected Mexican lime. This indicates that the p23 gene is involved in pathogenicity of CTV and symptom formation in citrus (Ghorbel et al. 2001). Since this gene induces symptoms in transgenic plants and no resistance has been reported in the transgenic

plants expressing the coat protein gene of CTV, it is necessary to evaluate other genes including those encoding replication-associated proteins as an alternative strategy to develop PDR to CTV.

Virus resistance induced by expression of native or modified forms of replication-associated genes, such as the RdRp of viruses in plants, is called replicase-mediated resistance (RMR). Since the reports of RMR against bacteriophage Q in bacteria (Inokuchi and Hirasima, 1987) and against TMV in tobacco (Golemboski et al. 1990), RMR has been extensively explored for many other plant RNA viruses. Transgenic plants resistant to viruses from *Alfamovirus*, *Bromovirus*, *Comovirus*, *Cucumovirus*, *Luteovirus*, *Potexvirus*, *Potyvirus*, *Tobamaovirus*, *Tombusvirus* and *Tobravirus* were developed using different constructs of their replicase genes (Palukaitis and Zaitlin 1997).

The replicase-mediated resistance to these viruses was achieved using different constructs of their replicase genes including the full length, un-translatable and defective RdRp genes. The resistance to TMV (Golemboski et al. 1990) pea early browning virus (PEBV) (MacFarlane and Davies,1992), cymbidium ringspot virus (CymRSV) (Rubino et al. 1993; Rubino and Russo, 1995), cowpea mosaic virus (CPMV) (Sijen et al. 1995), bromo mosaic virus (BMV) (Kaido et al. 1995), potato leaf roll virus (PLRV) (Kaniewski et al ., 1995), and rice tungro bacilliform virus (RTBV) (Huet et al. 1999) was developed by expressing their full-length RdRp genes. The defective constructs containing the truncated replicase lacking either the 3' or 5' terminus and the conserved GDD motif of the RdRp domain were used to engineer RMR to cucumber mosaic virus (CMV) (Anderson et al. 1992), TMV (Donson et al. 1993), alfalfa mosaic virus (AlMV) (Brederode et al. 1995) and potato virus Y (Audy et al.

1993). In addition, plants were transformed with mutant replicase genes with one or two amino acid changes in the conserved GDD motif of the RdRp domains of potato virus X (Longstaff et al. 1993 ; Mueller et al. 1995), AlMV (Brederode et al. 1995), plum pox virus (Guo and Garcia., 1997) to develop RM resistance to these viruses.

The analysis of the transgenic plants displaying RMR to some viruses demonstrated that the resistance operates at the single cell-level where the virus replication is inhibited (Carr and Zaitlin, 1991; Donson et al. 1993; Carr et al. 1994), and at the whole plant level where the cell-to-cell and/or long distance movement of the virus are restricted (Carr et al. 1994; Hellwald and Palukaitis, 1995; Nguyen et al. 1996; Gal-On et al. 1998). In the CMV resistant plants, long distance movement of CMV was completely inhibited by blocking the entry of the virus into the vascular system (Wintermantel et al. 1997). The RMR observed in transgenic plants can be expressed as either protein or RNA mediated resistance (Lommossoff, 1995; Baulcombe 1996; Palukaitis and Zaitlin 1997). Although the mechanisms of RMR were not studied in detail for some viruses, the RMR responses to TMV (Gloemboski., 1990; Donson et al. 1993), CMV (Anderson et al. 1992), PVY (Audy et al 1993), and AlMV (Brederode et al 1995) are at least in part similar to protein-mediated resistance. On the other hand, plants transformed with replicase genes of PVX (Muller et al. 1995), CpMV (Sijen et al. 1995), PMMV (Tenllado et al. 1996) and PPV (Guo and Garcia., 1997) showed characteristics typical of the RNA mediated resistance. Recent reports on the extensively studied RMR to TMV and CMV demonstrated that the resistance was contributed to both protein and RNA-mediated resistance mechanisms (Goregaoker et al. 2000; Wintermantel and Zaitlin, 2000), indicating that the RMR may be complex in some systems, and more than one factor can be responsible for the resistance phenotypes observed in transgenic plants.

In this study, five constructs (full-length, anti-sense, un-translatable and two modified constructs of RdRp gene with point mutations or a deletion in the conserved GDD motif) of the RdRp gene from a severe CTV isolate were prepared to test the possibilities of RMR against CTV. Epicotyl segments of Duncan grapefruit seedlings were transformed with these constructs using an *Agrobacterium*-mediated transformation method to regenerate grapefruit plants potentially resistant to CTV.

Materials and Methods

Cloning and Mutagenesis of CTV RdRp for Transformation

The RNA-dependent RNA polymerase gene of 3800, a grapefruit stem pitting isolate of CTV, was selected for transformation. The RdRp gene was amplified from a cDNA clone containing this region of the 3800 genome by polymerase chain reaction (PCR) using specific primers CN356 and CN357 (Table 6-1). Since the RdRp gene is expressed by a +1 translational frameshift and does not contain an ATG translational initiation codon, the ATG codon and a translational enhancer sequence (ACC) from CMV were incorporated into the 5' end of the RdRp sequence during PCR amplification. In addition, a *Not* I restriction site was integrated into both the 5' and 3' end of the sequence to facilitate cloning (Table 6-1). This full length sequence was designated as 3800 CTV RdRp and used as template for the generation of other constructs.

Table 6-1. List of primers used for PCR amplification, mutagenesis and sequencing of the RdRp constructs and the PCR amplification of the GUS gene.

Primer	Sequence (5' to 3')	Orientation
CN216*	CAACGAACTGAACTGGCAG	Sense
CN217*	CATCACCACGCTTGGGTG	Anti-sense
CN309	TGTTTTGTACCGGACCCTTA	Sense
CN310	GTACTCGCCTTCCATCCA	Anti-sense
CN355	*AAAGCGGCCGCAT*GAGAC**T**CTG**C**CCCT**AGT**GACTCCG GTAACTTA**G**ACGAACC	Sense
CN356	*AAAGCGGCCGCACCATGG*AGACACTGCCCCTCCCGACT CC	Sense
CN357	*AAAGCGGCCGC*TCAGCCGGTCGCTAAGTCGTCCG	Anti-sense
CN358	GTCCG**C**TG**C**TG**CTAGC**TTGATTTACTCCAAAAAAGG	Sense
CN359	CAA**GCTAG**CA**G**CA**G**CGACACGAGAAGTAACTCG	Anti-sense

Italic letters are non-CTV sequences.
Underlined letters indicate specific restriction sites incorporated into the primers.
Bold letters describes the mutations
* Indicates the GUS gene specific primers.

To produce an untranslatable construct of CTV RdRp that can potentially be used for RNA-mediated resistance, the 3800 RdRp was amplified by PCR using primers CN355 and CN357 (Table 6-1). Two nucleotides, AT, were added to the 5' end of the original RdRp sequence to form an out-of-frame translation initiation codon, ATG, with the first nucleotide, G, of the RdRp ORF. In addition, several point mutations were also made by oligonucleotide primer CN355 during PCR amplification to introduce three subsequent stop codons close to the 5' end of the CTV RdRp sequences. This resulted in an untranslatable sequence with *Not* I sites at both 3' and 5' ends which was designated as RdRp-UT (Figure 6-2A).

To produce a dysfunctional RdRp which may result in negative dominant mutations, the three amino acid GDD motif [glycine (G) , aspartic acid (D) and aspartic acid (D)] conserved in the CTV RdRp were mutated to alanine (AAA) by site directed mutagenesis using an overlap extension PCR method (Ho et al. 1989) (Figure 6-1A). First, two overlapping sense and anti-sense internal oligonucleotide primers CN358 and CN359 (Table 6-1) with the desired mutation were designed. They were used with an external primer CN356 or CN357 specific to the 5' and 3' ends of the RdRp gene for PCR amplification of two overlapping DNA fragments from the 3800 CTV-RdRp. Then, these DNA fragments were mixed and used as template for second round of PCR amplification using only the external primers (CN356 and CN357) to produce the complete RdRp sequence with the desired mutations, which was designated as RdRp-mGDD (Figure 6-1A and 6-2B).

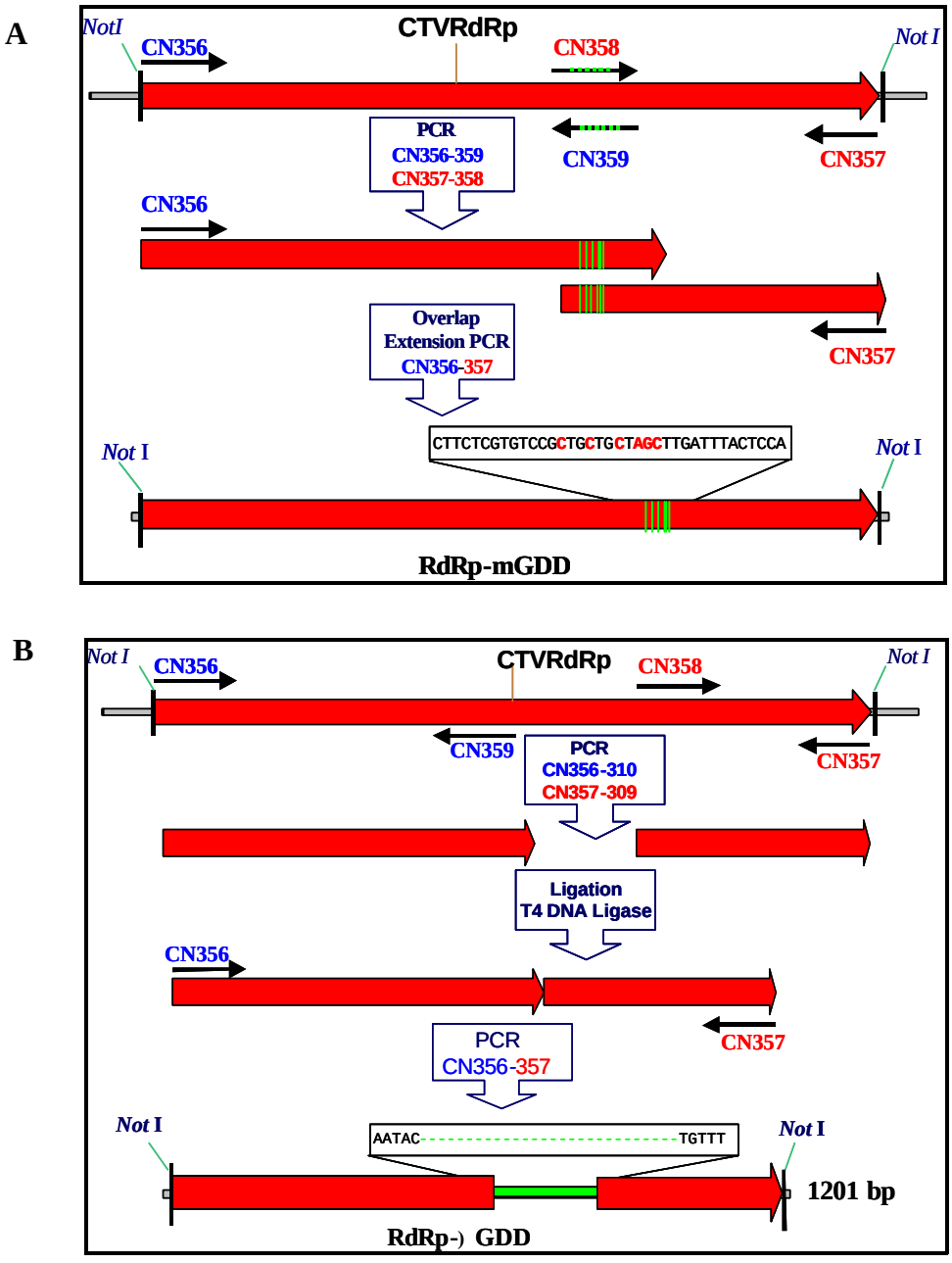

Figure 6-1. Description and flow chart of the site-directed mutagenesis of the RdRp gene of CTV (A) The overlap extension PCR for mutagenesis of the conserved GDD motif of CTV RdRp to AAA (B) The ligation mediated PCR for deletion of about 300 nucleotide sequence near the GDD coding region of the CTV RdRp gene. The point mutations and deletion are shown by vertical and horizontal green lines, respectively. The location and orientation of the primers are indicated with black arrows.

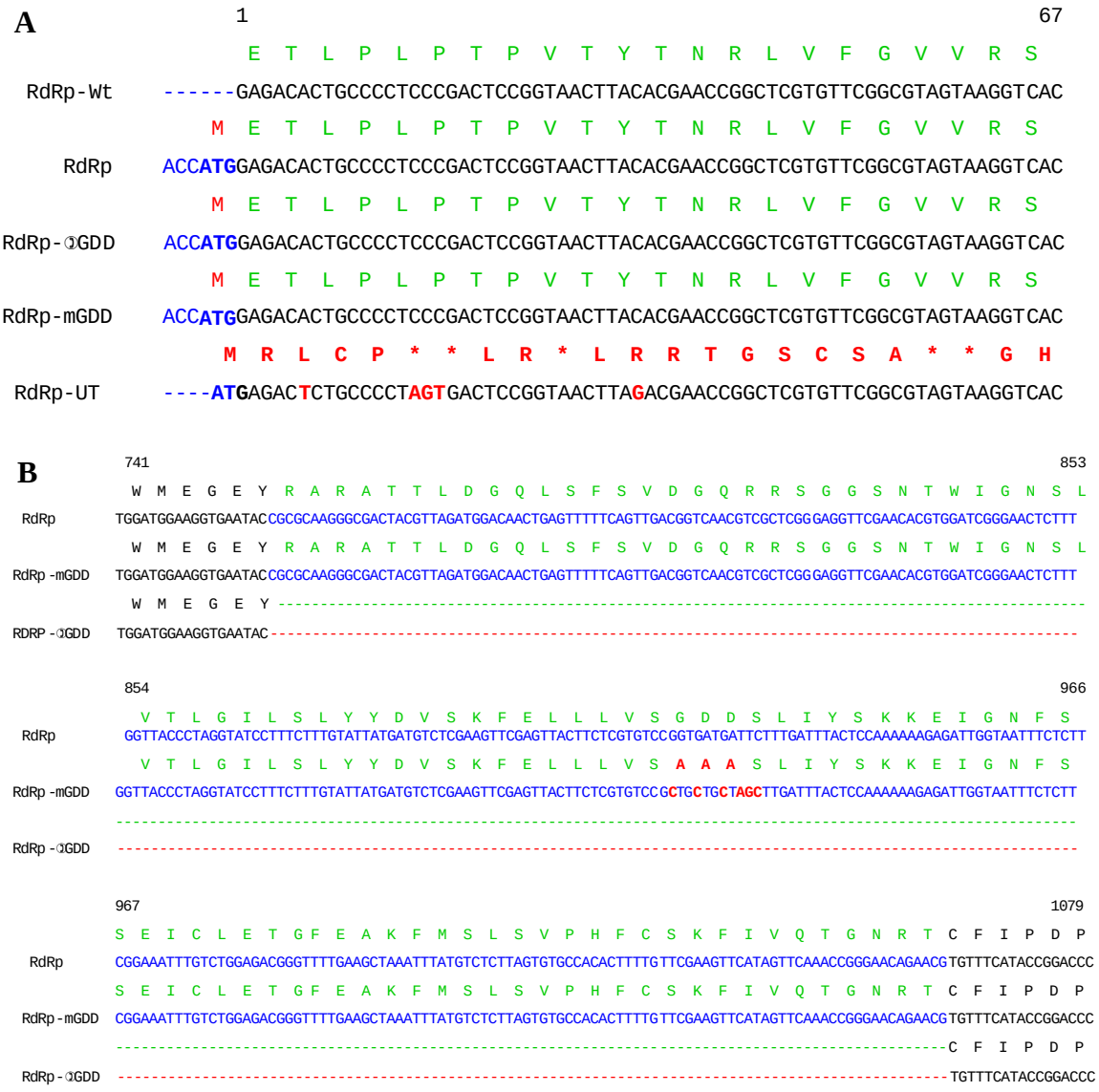

Figure 6-2. Partial nucleotide and amino acid sequence alignments of CTV-RdRp constructs showing the deletion and point mutations made in the RdRp gene. (A) The alignment of the 5' end of the wild type RdRp (wt) gene of CTV and that of four plant transformation constructs described in the text. (B) The alignments of the plant transformation constructs showing site directed mutations in the GDD motif and the 300-nt deletion in that region. Wild type CTV nucleotide and amino acid sequences are shown in black and green, respectively. Changes from the wild type nucleotide and amino acid sequences are shown in red. Asterisks and dashes indicate stop codon and deleted sequences, respectively.

To produce another dysfunctional RdRp which may function as a negative dominant mutant, a 300 bp nucleotide sequence containing the region coding for the GDD motif was deleted using a ligation-mediated PCR method. First, one anti-sense (CN310) and sense (CN309) in-frame internal primers located up- and down-stream of the GDD coding region, respectively, were selected from the sequencing primers described in Chapter 2. They were used with an external primer CN356 or CN357 specific to the 5' and 3' ends of the RdRp gene for PCR amplification of two DNA fragments from 3800 CTV-RdRp. These DNA fragments were mixed and ligated using T4 DNA ligase. The ligation mixture was then used as template for second round of PCR amplification using the external primers (CN356 and CN357) to amplify about 1200 bp of the RdRp gene with the desired 300 bp deletion around the GDD coding region, which was designated as RdRp- GDD (Figures 6-1B and 6-2B).

A pUC118-based plasmid vector containing the cauliflower mosaic virus 35S promoter and termination signal (pUC118 CaMVP-T), which was kindly provided by Dr. Vicente Febres, was used for generation of the RdRp plant transformation constructs. First, the PCR amplified and modified CTV RdRp sequences were cloned into a *Not* I site between the CaMV 35S promoter and the termination signal. The resulting clones of pUC118 CaMVP-T with CTV RdRp constructs were digested with *Pst* I restriction endonuclease to release the CTV RdRp sequences with the CaMV 35S promoter and the termination signal. These fragments were cloned into the *Pst* I site in the binary plant transformation vector pCambia 2203 with the Npt II gene as selectable marker and GUS and green fluorescent protein (GFP) as the bi-functional reporter genes (Figure 6-3). The pCambia 2203 binary vectors with the CTV-RdRp constructs were introduced into *Agrobacterium tumefaciens* strain Agl I using the cold shock transformation method.

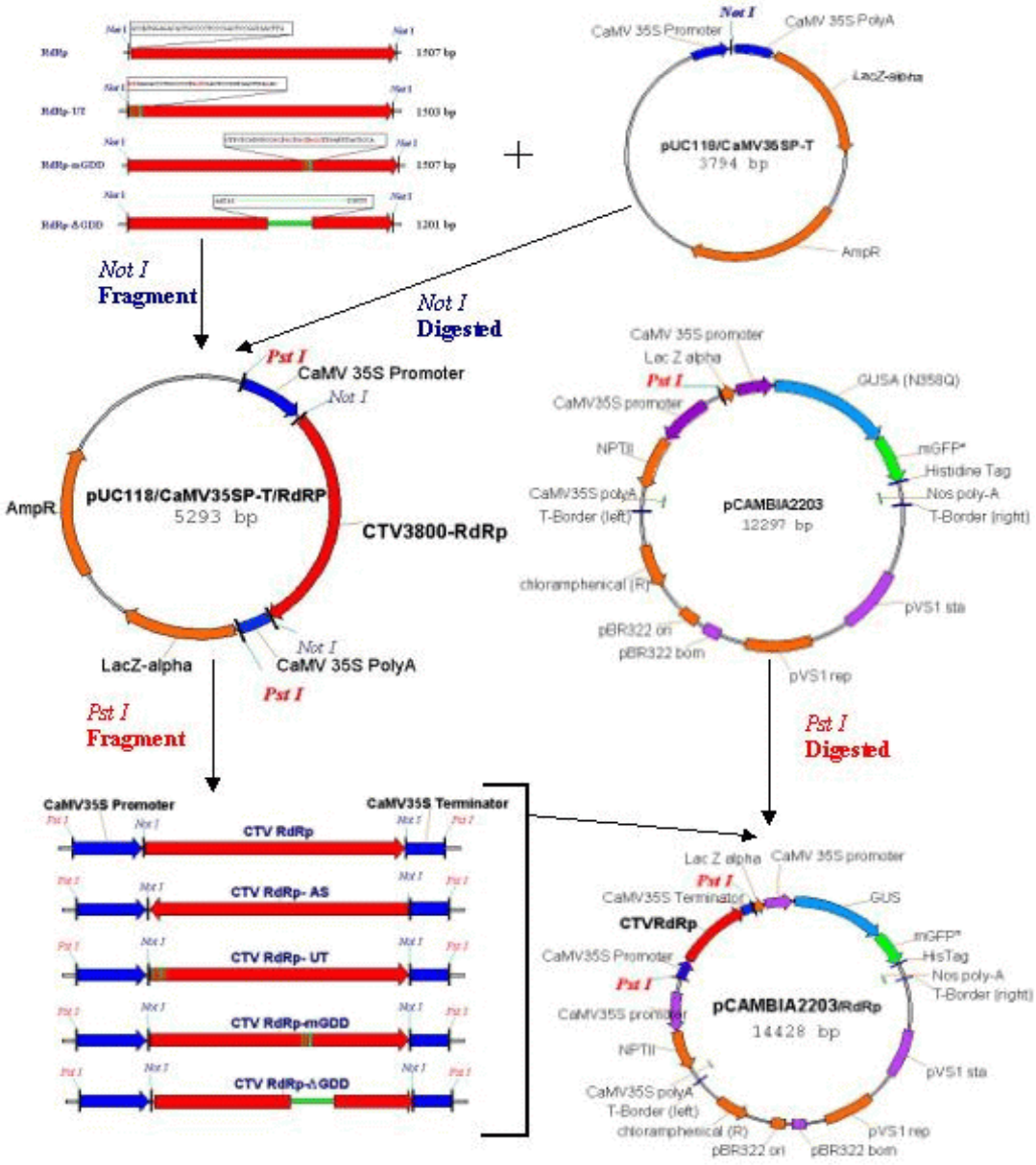

Figure 6-3. Cloning of the CTV-RdRp constructs for plant transformation. The PCR generated RdRp sequences were cloned into a *Not* I site between the CaMV 35S promoter and the termination signal. The resulting clones of pUC118 CaMVP-T with CTV RdRp constructs were cloned into the *Pst* I site in the binary plant transformation vector pCambia 2203.

Genetic Transformation of *Citrus* with RdRp gene of CTV

An *Agrobacterium*-mediated transformation protocol previously developed for epicotyl segments from rootstock cultivars Carrizo citrange or Swingle citrumelo (Moore et al. 1992; Moore et al. 1993) and recently improved and applied to the etiolated seedlings of *Citrus paradisi* cv. Duncan by Luth and Moore (1999) was used for all stages of transformation and regeneration of transgenic plants in this study. The steps of this transformation method are briefly described here.

Seed germination

Citrus paradisi cv. Duncan seeds were peeled and sterilized first with 70% ethanol for 5 min and 0.525% hypochlorite solution plus 0.05% Tween-20 for 10 min, then they were rinsed five times with sterile distilled water. The seeds were placed individually into 150 X 25 mm tubes containing half-strength MS medium (2.13 g/l MS salt, 50 mg/l myo-inositol, 15 g/l sucrose and , pH 5.7) with 7 g/l agar. The tubes were kept in the dark at 28 ^{0}C or at room temperature until the germinated seedlings were used for transformation experiments, approximately 4–6 weeks after planting (Figure 6-4A).

Transformation of epicotyl segments

Agrobacterium tumefaciens strain Agl I containing binary plasmid pCambia2203 with CTV RdRp constructs was inoculated into YEP medium (10 g/l Bactopeptone, 10 g/l yeast extract and 5 g/l NaCl pH 7.0) containing the appropriate antibiotics. They were grown overnight to log phase (OD_{600}nm = 0.5-1.0) at 280 rpm and 28 ^{0}C. The cultures were centrifuged at 4 ^{0}C and 5000 rpm for 5 min, and the pellets were resuspended to a final concentration of $5x10^8$ cfu/ml in MS medium with 100 µM acetosyringone.

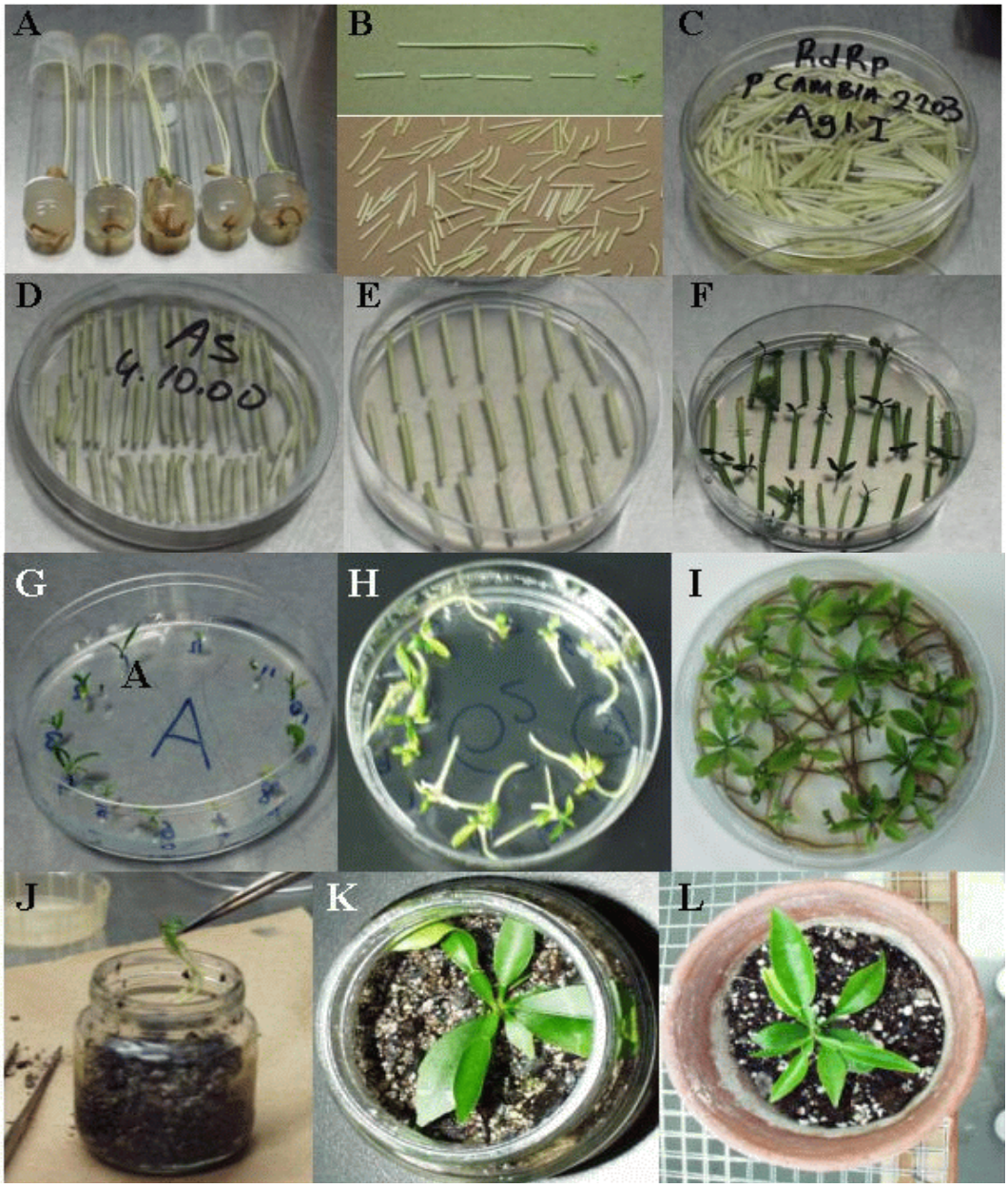

Figure 6-4. Production of transgenic grapefruit plants from epicotyl segments using *Agrobacterium*-mediated transformation method (A) *In vitro* production of etiolated seedlings (B) Preparation of etiolated seedling segments for transformation (C) Inoculation and (D) Co-cultivation of epicotyl segments with *Agrobacterium* strain AGL1 containing the binary plasmid with CTV-RdRp construct. (E-F) Regeneration and selection of transgenic shoots using benzyl adenine and kanamycin sulfate. (G-I) Rooting of the transgenic shoots in naphthalene acetic acid-containing medium. (J) Transfer of rooted transgenic shoots into the soil jars. Transgenic plant in a soil cup (K) and (L) in a pot in the greenhouse.

The epicotyl portions of the etiolated seedlings were cut into 1 cm segments and soaked in the *Agrobacterium* inoculum for 1 min. (Figure 6-4C). The inoculated segments were placed horizontally in petri plates containing co-cultivation medium (MS medium plus 7 g/l agar, and 100 µM acetosyringone)(Figure 6-4D). The plates were sealed and kept in the dark at room temperature for 2–3 days.

Selection and regeneration of transgenic shoots

After 2-3 days of co-cultivation, the epicotyl segments were transferred to a shoot induction medium (MS medium with 0.5-2.0 mg/l benzyl adenine (BA) and 7g/Bacto-agar) supplemented with 500 mg/l Claforan to inhibit further growth of *Agrobacterium* and with 75 mg/l kanamycin sulfate for selection of transgenic shoots (Figure 6-4E and F). The plates were maintained at 28 ^{0}C with a 16-h photo-period provided by cool-white fluorescent light for 4-5 weeks.

Rooting of transgenic shoots

When shoots appeared and reached about 5-10 mm length, they were removed from the explants, and a small sections were cut from their basal ends for histochemical GUS staining. The shoots grown in high (>1 mg/l) BA were placed into petri plates containing antibiotic- and hormone-free MS medium and maintained there for 2- 3 weeks. Fresh cuts were made on basal ends of the stems before they were transferred onto root induction medium (MS medium with 0.5 mg/l naphthalene acetic acid (NAA). The shoots grown in low (<1 mg/l) BA were removed from the explants and after cutting their basal ends for histochemical GUS staining, they were placed directly on rooting medium. Shoots remained on this medium for 3-5 weeks or until they produced roots (Figure 6-4G and H).

Shoots formed 1–3 roots, and after the root was at least 1cm long, the plants were transferred to sealed culture jars containing sterilized soil and half-strength MS. The jars were placed in a growth chamber at 28 ^{0}C with a 16-h photo period or at room temperature until they grew out of the soil jars (Figure 6-4 J and K). Surviving plants were removed from the jars and transferred into pots with soil and covered with small plastic bags or clear plastic cups. Based on the availability of the space, these pots were either placed in a high humidity growth room at 30 ^{0}C and a 16-h photo period and 28 ^{0}C and a dark cycle or in the greenhouse. The plants in the high humidity growth room were later moved to the greenhouse and maintained there (Figure 6-4K and Figure 6-6A).

Analysis of Transgenic Shoots and Plants

Flourescent microscopy

In the initial experiments, the epicotyl segments transformed with *Agrobacterium* containing the CTV RdRp constructs and shoots regenerated from these segments were periodically examined for the expression of GFP using a dissecting microscope (Zeiss) as described in Chapter 5.

-glucuronidase (GUS) assay

Regenerated shoots were removed from the epicotyl segments in the selection medium and small sections of about 1 to 2 mm were cut from the basal end of the shoots. They were places in a 96-well assay plates containing -glucuronidase assay solutions (50 mM $NaPO_4$ pH 7.0 10 mM Na_2EDTA and 5 mg/ml 5-bromo-4-chloro-3-indolyl- -D-glucuronide). A mild vacuum was applied to the plates for 5 min to infiltrate the substrate equally into the tissue. The plates were sealed and incubated overnight at 37 ^{0}C . The shoot sections were cleared using a solution of 70% ethanol and 30% acetic acid and analyzed for the GUS staining (Figure 6-5C to E). The GUS stained sections were examined under a stereo-

microscope and scored for GUS staining and classified as partial GUS positives with a few blue dots (Figure 6-5D) or solid GUS positives showing blue staining in the whole section tested (Figure 6-5E). Leaves from some of the plants showing GUS staining were tested for GUS activity a second time when they were transplanted into pots. A small young leaf or a portion of a larger young leaf was cut from each plant and placed in a 1.5 ml microfuge tube containing -glucuronidase assay solutions, and mild vacuum was applied to the samples. After overnight incubation at 37 C leaves were cleared and evaluated for GUS histochemical stainings (Figure 6-5 A and B) as described above.

Polymerase Chain Reaction Assay

The putative transgenic plants were tested for the presence of the GUS and CTV-RdRp genes using polymerase chain reaction with gene-specific primers. Ten GUS positive transgenic plants which repeatedly showed solid blue staining were selected for initial PCR analysis. The genomic DNA was isolated from these plants using DNAzol reagent (Gibco-BRL) according to the manufacture's instructions and/or previously reported rapid DNA extraction methods. The DNA from these plants were tested by PCR using two sets of primers: one specific to the the GUS gene (CN 216 and CN 217) and the second one specific to the 5' half of the RdRp gene of CTV (Table 6-1) using Taq DNA polymerase. The amplification reaction was carried out in a Biometra thermocycler using a profile of 94 °C for 3 min initial denaturation and 40 cycles of denaturation at 92 °C for 30 min, primer annealing at 55 °C for1 min, and primer extension at 72 °C for 1 min followed by final primer extension at 72 °C for 5 min. The amplification products were separated in 1 agarose gels by electrophoresis in TAE buffer and analyzed by ethidium bromide staining.

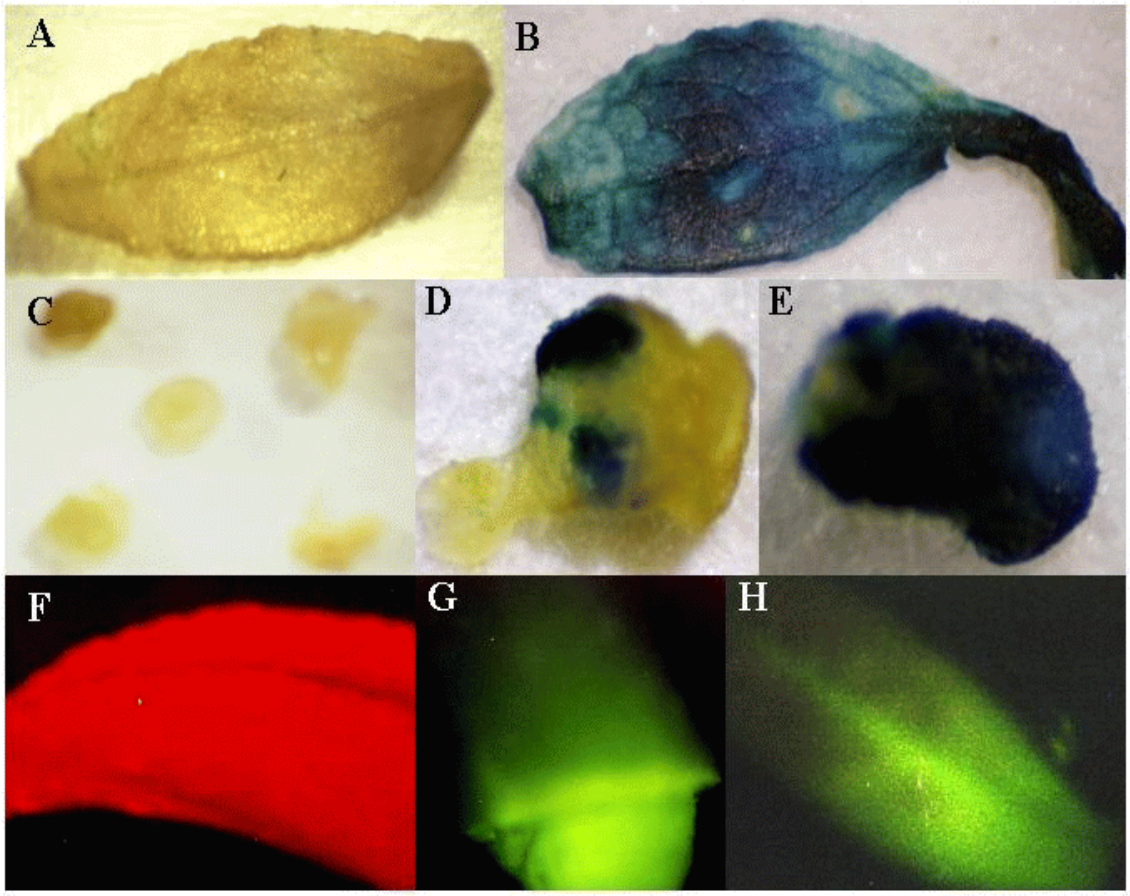

Figure 6-5. Analysis of the transgenic shoots regenerated from epicotyl segments transformed with *Agrobacterium tumefaciences* carrying pCambia2203CTV-RdRp constructs. Histochemical staining of leaves from non-transgenic (A) and transgenic plants (B). The basal end of non-transgenic shoots (C) and putative transgenic shoots showing partial (D) and solid (E) GUS stainings. Fluorescent microscopy images of leaves from a non-transgenic shoot (F) transformed stem segment (G) and a leaf of potentially transgenic plants with the RdRp-mGDD construct.

Once the PCR was optimized, the genomic DNA was isolated using about 1 cm^2 leaf tissue from all putative transgenic plants using the rapid genomic DNA extraction methods reported for mapping in *Arabidopsis* (Edward et al. 1991) and/or citrus (Oliveira et al. 2000) and tested for the presence of the GUS and the RdRp genes.

Results and Discussion

Regeneration of Transgenic Plants

A total of 7,660 epicotyl segments were transformed with *Agrobacterium tumefaciences* strain AglI carrying the binary plasmid pCambia 2303 with the five different constructs of the CTV RdRp gene. More than 30% of these segments produced kanamycin resistant shoots resulting in 2,442 total shoots for all five constructs. The average number of shoots per segment was similar to the number previously reported for grapefruit (Luth and Moore, 1999). The percentage of shoot regeneration for the RdRp (41%) and the RdRp-AS (41%) constructs was higher than the other three constructs (25 to 27%), because 1.5 mg/l of BA was used for shoot regeneration in the initial experiments with RdRp and RdRp-AS constructs. Although the high concentration of BA (1.5 mg/l) produced more shoots per segment, most of the shoots regenerated did not root, even when they were kept on antibiotic and hormone-free MS medium for a month and then transferred to the rooting medium. Therefore, the BA concentrations was reduced to 0.5 mg/l in the later experiments with all five constructs to improve the rooting efficiency. The results for transformation experiments with individual RdRp constructs are summarized in Table 6-2.

Table 6-2. Summary of transformation experiments with five different constructs of CTV-RdRp gene.

Constructs	Number of segments	Regenerated shoots		Number of GUS positive shoots		% GUS positive shoots		Number of shoots	
		Total	%*	Total	Solid	Total	Solid	Rooted	Potted
RdRp	1220	511	41.9	64	9	12.5	14.1	31	22
RdRp-AS	1360	546	40.2	59	8	10.8	13.6	36	24
RdRp-mGDD	1640	459	28.0	69	10	15.0	14.5	51	37
RdRp-UT	1760	494	28.1	72	12	14.6	16.7	64	42
RdRp- GDD	1680	432	25.7	68	11	1 5.7	16.2	54	38
Total	7660	2442	31.9	332	50	13.6	15.5	236	163

* The percentage of shooting was calculated using the total number of shoots regenerated from the total number of segment.

During regeneration, transformed segments and shoots regenerated on these segments were examined periodically for the expression of GFP using a dissecting flourescent microscope. The expression of the GFP was detected in some segments (Figure 6-5G) and shoots (Figure 6-5 H); however, the detection of the GFP expression was not consistent and was blocked by strong autofluorescence in the citrus leaf (Figure 6-5F). Therefore, fluorescent microscopy was not used for scoring transgenic plants; instead they were determined by histochemical GUS assays.

A section cut from basal end of all 2,442 regenerated shoots was tested for GUS activity by histochemical staining assay before the shoots were transferred to the rooting media. Among them, 332 (13.6 %) showed GUS staining (Table 6-2) ranging from a few blue dots (Figure 6-5D) to completely blue stained cuts (Figure 6-5E), and they were considered as putative transgenic shoots. The sections from remaining shoots did not have any visible GUS staining under dissecting microscope, and no GUS staining was observed on the section from untransformed shoots (Figure 6-5C). Based on the degree of the staining shoots were classified as solid GUS positives if they complete blue staining (Figure 6-5E) and partial GUS positive with a few blue stained cells (Figure 6-5D). The majority of the shoots tested showed partial GUS staining, and only 15% of total GUS positive shoots showed solid blue staining. The results of histochemical staining for individual constructs are summarized in Table 6-2. The efficiency of transformation in citrus was generally determined by the number or percentage of GUS+ shoots from the total number of shoots regenerated from epicotyl segments (Gutierrez et al. 1997; Luth and Moore,1999) or number or percentage of epicotyl segments producing GUS + shoots (Bond and Roose, 1998; Cervera et al. 1998). Since a large number of epicotyl segments were used in this study, the transformation efficiency was

determined by percentage of GUS + shoots from the total shoots tested. As shown in Table 6-2, the overall efficiency of transformation for this study was 13.5 %, and it ranged from 10.8 % (RdRp-AS) to 15.7% (RdRp- GDD) for individual constructs. The percent GUS + shoots was lower in this study (13.9%) than the previous reports on transformation of other citrus including citrange (Gutierrez et al. 1997; Cervera et al. 1998) Mexican lime (Gutierrez et al. 1997; Pena et al. 1997), and sweet orange (Bond and Roose, 1998). Since it has been reported that different citrus species and even cultivars of citrus are transformed with different efficiencies, the variation in the transformation frequency of grapefruit in this study and previous reports on citrange, Mexican lime and sweet orange (Gutierrez et al. 1997; Pena et al. 1997; Cervera et al. 1998) may be due to differences in the type of citrus and transformation and regeneration techniques used in each study. The total GUS+ shoots in this study also was lower than in the previous report for *Agrobacterium mediated* transformation of Duncan grapefruit (Luth and Moore, 1999) even though the same method was used in this study. However, the percentage of solid GUS+ in this study (15%) was higher than the previous report by Luth and Moore (1999). Although the same citrus cultivar and transformation technique were used in both studies, different *Agrobacterium* strains and binary plasmids were used in the previous report of grapefruit transformation. It was reported that a wide spectrum of variation can be observed with different stain of *Agrobacterium* in citrus and other plants suggesting that variation in the transformation frequency of grapefruit in this study and the previous one may be due to the use of different *Agrobacterium* strain.

The regenerated shoots were rooted on MS media containing 0.5 mg/ml of NAA, and rooted GUS positive plants were transferred into soil insmall sterile glass jars with lids (Figure 6-4 J and K). A total of 332 GUS + shoots were placed into rooting media, and 71%

of them rooted and were transferred into soil jars. The remaining shoots did not survive or did not root. The number of rooted GUS+ plants was higher for constructs RdRp-mGDD, -UT and - GDD than for theRdRp and RdRp-AS. The results are summarized in Table 6-2. As discussed above, the difference was due to the higher concentration of BA used for some of the initial experiments with these two constructs which reduced the rooting efficiency of transgenic shoots, and more GUS+ shoots from these constructs did not root.

In one to three months or when the surviving plants grew out of the jars, they were transplanted into non-sterile pots with soil mixture and maintained in the greenhouse (Figure 6-4 K and L , and Figure 6-6 A). A total of 163 plants were potted and transferred to the greenhouse (Table 6-2) and most of these plants survived and are still maintained in the greenhouse. There are now more than ten putative transgenic lines established and maintained in the greenhouse from individual CTV-RdRp construct. Buds from some of these plants were micro-grafted onto sour orange rootstock for duplication and testing for CTV resistance by either graft inoculation of infected tissue or aphid transmission.

Analysis of Transgenic Plants by Polymerase Chain Reaction Analysis

First, ten putative transgenic plants which repeatedly showed solid GUS staining were selected for PCR analysis. The genomic DNA was isolated and tested for the presence of the GUS and the RdRp genes using primers specific to these genes. An 800 bp fragmentwas amplified from all ten putative transgenic plants and the control pCambia 22

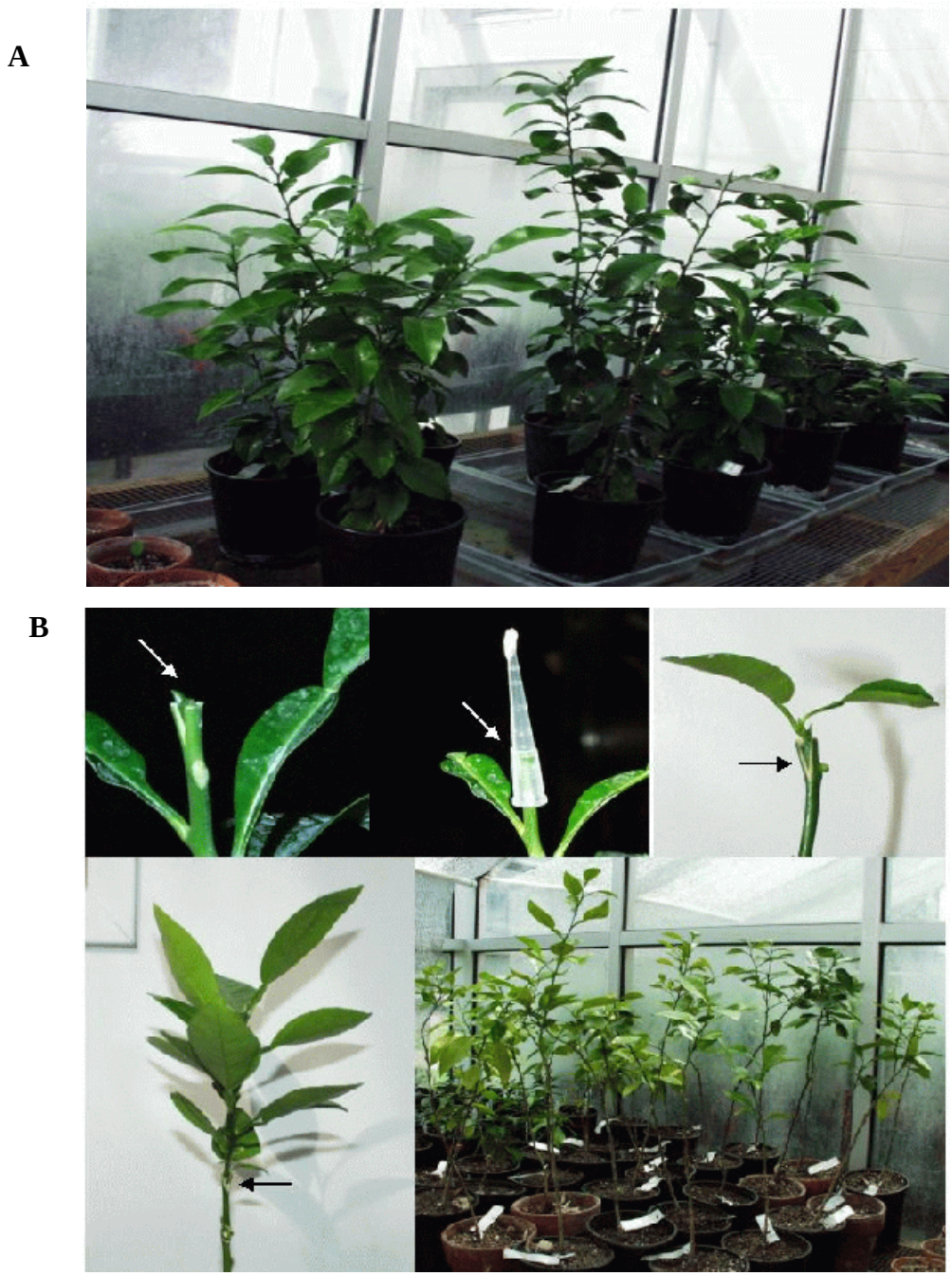

Figure 6-6. The transgenic plants with different constructs of the CTV-RdRp gene. (A). Some of the potted GUS+ and PCR+ transgenic plants in the greenhouse. (B) Micro-grafting of the transgenic grapefruit plants on sour orange rootstock for multiplication and testing for CTV resistance. The arrows indicate the graft union of the scion and the rootstock.

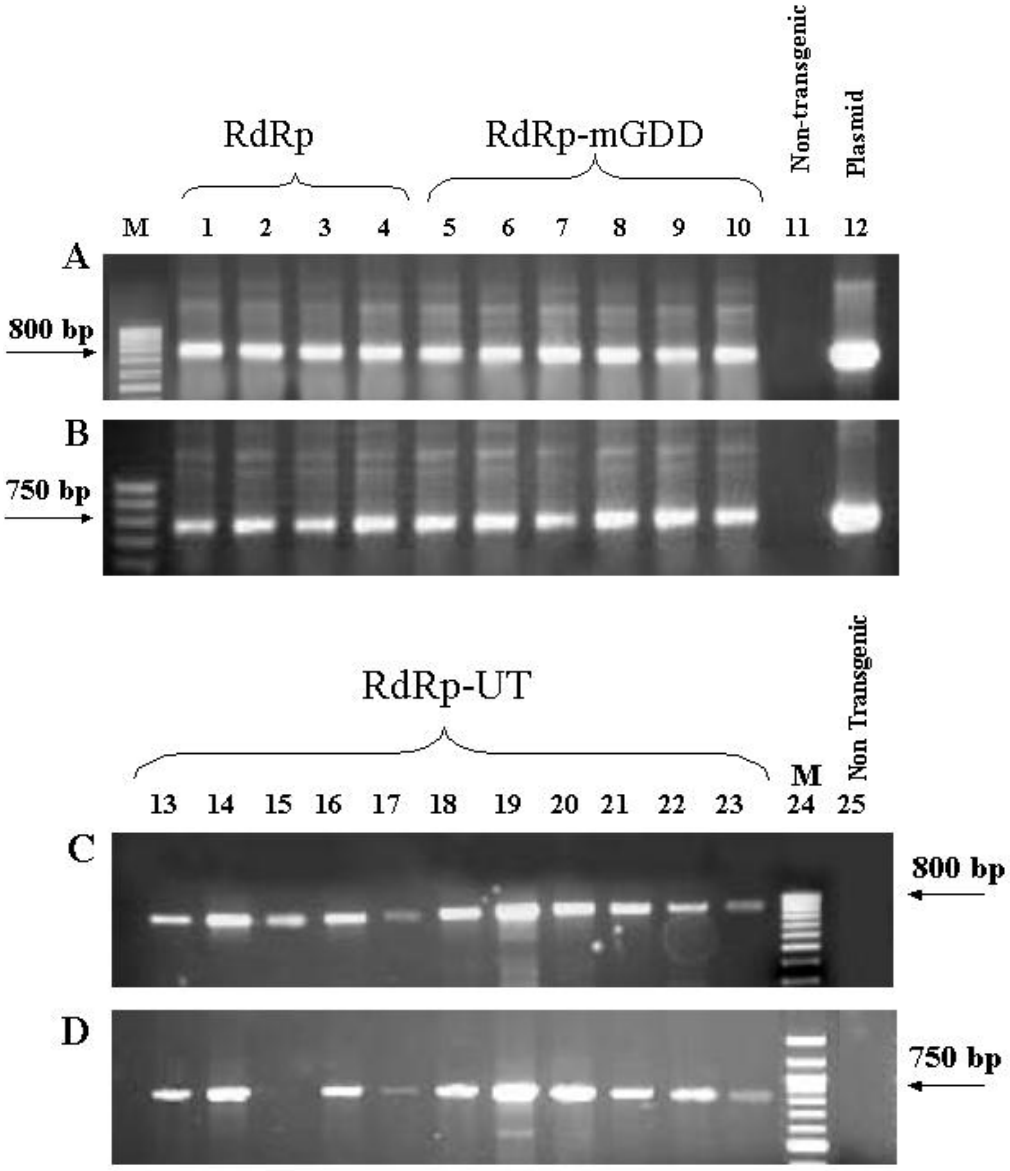

Figure 6-7. Analysis of representative putative transgenic plants by polymerase chain reaction. Optimization of amplification of the GUS gene using primers CN216 and 217 (A) and the RdRp gene using primers CN356-310 (B) from genomic DNA isolated from plants transformed with the RdRp and RdRp-GDD constructs using DNAzol reagent. Screening of putative transgenic plants for the GUS gene using primers CN216 and 217 (C) and the RdRp gene using primers CN356-310 (D) from the genomic DNA isolated from plants transformed with RdRp-UT construct using rapid extraction methods.

transformation method. Since no amplification was detected in the samples from non-transformed plants (Figure 6-7A and B), these fragments were considered to be gene specific.

Once the PCR assay was optimized, genomic DNA from 92 additional GUS+ plants (solid or partial) were isolated and tested for amplification of GUS and RdRp genes using specific primers (Figure 6-7 C and D). Among the 112 GUS+ plants tested, the 800 bp fragment of GUS gene and the 750 bp fragment of CTV RdRp gene was amplified from 79 and 78 individual plants, respectively. Both the GUS and the RdRp genes were amplified from 69 plants, and neither the GUS nor the RdRp gene was amplified from 15 plants indicating that the plants were false positives or their DNA was not good for PCR. The remaining 28 plants showed differential amplification of the GUS and RdRp genes meaning that only the GUS gene was amplified from 15 plants and only the RdRp gene was amplified from 13 plants. These results were confirmed by two or more PCR amplifications from these plants. The number of plants analyzed by PCR for each construct and the results of PCR analysis are summarized in Table 6-3.

Differential detection of the GUS and RdRp genes may simply be due to false positive amplification of the one of the genes or the result of partial integration of T-DNA into the plant genome. It was reported that the transfer of T-DNA from *Agrobacterium* to the plant cell starts at the right border (RB) and continues toward the left border (LB) (Sheng and Citovsky, 1996). Any interruption during the T-DNA transfer may result of partial transfer of T-DNA region close to the RB where the GUS gene of pCambia 2203 is located. A polarity may also be observed during T-DNA integration into the plant genome, leading to insertion of only a part of T-DNA into the plant genome. Although partial integration may

Table 6-3. Summary of the PCR analysis of the transgenic plants with different constructs of CTV RdRp gene.

Constructs	Number of Potted Plants	PCR Analysis						
		Tested	Total GUS +	Total RdRp +	GUS+ RdRp+	GUS+ RdRp -	GUS - RdRp+	GUS - RdRp -
RdRp	22	18	16	16	15	1	1	1
RdRp-AS	24	18	11	12	8	4	4	2
RdRp-mGDD	37	32	20	20	21	2	3	5
RdRp-UT	42	23	15	12	10	6	1	6
RdRp- GDD	38	21	17	18	15	2	3	1
Total	163	112	79	78	69	15	13	15

explain the differential amplification of the GUS and RdRp genes in some transgenic plants, PCR amplification by itself does not conclusively prove the integration of the GUS and RdRp gene into the plant genome. Transgenic plants should be further analyzed by Southern blot to conclusively demonstrate the integration of the T-DNA into the these plants and to determine the copy number of transgenes introduced into the genomes of the individual plants. In addition, the expression of transgene should be determined by Northern and/or Western blot analysis.

Conclusions

In attempts to develop pathogen-derived resistance against CTV, five different constructs of the RdRp gene of CTV, including full length, untranslatable, antisense and two modified RdRp genes with GDD to AAA mutation and deletion around the conserved GDD motif were cloned under the control of CaMV 35S promoter. Duncan grapefruit seedling segments were transformed with these constructs using an *Agrobacterium*-mediated transformation method. Transgenic shoots with GUS reporter gene activity were regenerated and whole plants transformed with individual RdRp constructs were established in the greenhouse. These plants were analyzed by PCR, and the transgenic nature of most plants in the greenhouse were confirmed by amplification of the GUS and the RdRp genes from the genomic DNA of these plants. Transgenic plants have not yet been analyzed by Southern blot analysis to determine the copy number of the transgenes and by Northern or Western blot analyses to study the expression of transgenes in transgenic plants produced in this study. These plants need to be more throughly analyzed by these methods and also tested for resistance to CTV.

CHAPTER 7
SUMMARY AND CONCLUSIONS

The RNA-dependent RNA polymerase (RdRp) is required for the replication of all RNA viruses. Characterization of the RdRp is necessary for a better understanding the mechanism of viral replication and developing strategies to interfere with the virus life cycle to control or prevent virus infection.

Citrus tristeza virus is an economically important virus which cause serious diseases in citrus worldwide. Characterization of CTV has been slow due to its large genome, complex host relations and pathogenicity. Molecular characterization of CTV genome and a better understanding of its gene expression and function, as well as our knowledge of the diversity of CTV isolates have advanced dramatically in recent years. Since the ORF 1b of CTV encodes the RdRp which is required for CTV replication, characterization and understanding of the expression of this gene will contribute to the development of better control strategies against CTV.

To determine the sequence variation within the RdRp gene and to study the relationship among geographically and biologically different isolates of CTV, the RdRp genes from distinct isolates of CTV were amplified by RT-PCR using primers specific for highly conserved regions of the genome. The PCR products were used for direct sequencing to determine major sequence populations in the infected plants. Sequence analysis revealed that

the RdRp genes of biologically and geographically different isolates of CTV are highly conserved in their nucleotide and encoded amino acid sequences. The most obvious difference among the RdRp genes of CTV isolates is the presence of an 18-nucleotide in-frame insertion sequence close to the 3' end of the RdRp genes of isolates T36 and T66, both QD isolates from Florida. At this point, the insertion sequence is rare among CTV isolates, and it is not associated with all QD isolates. Comparative sequence analysis demonstrated that the QD isolate T36 from Florida was the most different isolate. It was also found that the RdRp genes of some CTV isolates with similar biological activity have a significantly higher level of sequence identity, indicating that they are more closely related at the molecular level. Based on phylogenetic analyses of their RdRp genes, CTV isolates examined can be divided into four distinct groups. Three of these groups are interrelated and possibly diverge from the same lineage. However, the group I is more distantly related to these groups, suggesting that it may be from a different lineage. The group I includes only QD isolates, however, the other groups contain mild, seedling yellows and stem-pitting isolates. Within the group II, biologically related isolates of CTV are clustered into smaller sub-groups. Since CTV isolates show unusual and asymmetric sequence relationships along their genomes, comparison of the RdRp genes sequences may provide the most straightforward method for phylogenetic analysis

The RdRp gene of T36 was successfully expressed in *E.coli*, and a polyclonal antiserum specific to the RdRp was produced. A 50 kDa protein was detected in CTV-infected tissue using these polyclonal antiserum produced against the recombinant RdRp expressed protein. Using a cell fractionation method, it was found that the 50 kDa protein was localized in the cytoplasmic and membrane enriched fractions of infected tissue, and mostly in the membrane fraction. Since replication-associated proteins of other RNA viruses

are also localized in the membranes, the protein detected by this antiserum most likely is the RdRp of CTV. It can be concluded from this result that the CTV-RdRp gene is expressed in the infected plants indicating and proposed +1 translational frameshift does occur in the CTV infected tissue. The size of the protein detected in infected plants is significantly smaller than the maximum size of the CTV polyprotein indicating that after the expression of RdRp as a fusion protein within the CTV polyprotein, it is processed and cleaved from the rest of the polyprotein encoded by CTV ORF1a by either host and/or CTV protease(s).

The requirement of the +1 frameshift for the expression of RdRp gene of CTV was demonstrated using a coupled *in vitro* transcription and translation assay. This assay indicated that the efficiency of the +1 translational frameshift was about 1-5% . An *Agrobacterium*-mediated transient expression assay was adopted and used to analyze the effect of the overlap region of ORF1a and 1b of CTV in the +1 frameshift. Using the transient expression assay, it was demonstrated that the overlap region of ORF1a and 1b of CTV promotes the +1 frameshift that is required for the expression of the CTV RdRp gene. It was shown that the 123 nt overlap sequence itself is necessary and sufficient to induce the +1 frameshift as indicated by the expression of both the GUS and GFP reporter genes cloned into the +1 frame position in relation to the ATG translation initiation codon.

Sequence analyses of the overlap region of the CTV ORF1a and 1b revealed that the region is highly conserved and a similar stem-loop secondary structure was present in the sequences from ten different isolates of CTV. Site directed mutagenesis in this region demonstrated that any mutations affecting the secondary structure eliminated the +1 frameshift, indicating that the stem-loop structure was required for the frameshift. On the other hand, it was found the mutation of the rare arginine (CGG) to more frequent arginine codon (AGG) did not have any effect on the frameshift, indicating that it was not required for or involved in the

+1 frameshift. Although the actual specific nucleotides required for the +1 frameshift were not identified, it was clearly shown by both *in vitro* translation and the *Agrobacterium*-mediated transient assay that the RdRp gene of CTV is expressed by the +1 frameshift induced by the overlap sequences of CTV ORF1a and 1b. Since it has been reported that the +1 frameshift of Ty-1 in yeast is specifically inhibited by pokeweed antiviral protein, the inhibition of the +1 frameshift with this protein or other molecules or genes provides a specific target for engineering resistance to CTV.

To explore the possibilities of replicase mediated pathogen-derived resistance against CTV, five different constructs of the CTV RdRp gene were made, including full length, untranslatable, anti-sense and two modified RdRp gene with mutations and deletions at the GDD motif were prepared. Following transformation of Duncan grapefruit seedling segments using *Agrobacterium* containing these constructs, a number of transgenic plants were regenerated and established in the greenhouse. These plants were analyzed by PCR using specific primers for amplification of the GUS and CTV-RdRp genes, and the transgenic nature of most plants in the greenhouse was confirmed. Transgenic plants should be further analyzed by Southern blot to determine the copy number of the transgenes and/or by Northern and Western blots to study the expression of transgenes. Since these plants were transformed with five different constructs that were designed to induce protein or RNA-mediated forms of replicase-mediated resistance against CTV, it is possible that some of this transgenic plants may be resistant to CTV.

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BIOGRAPHICAL SKETCH

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