To my family
ACKNOWLEDGMENTS

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<tr>
<td>ATG8</td>
<td>Autophagy 8</td>
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<tr>
<td>BrCA</td>
<td>Brown citrus aphid</td>
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<tr>
<td>CaMV</td>
<td><em>Cauliflower mosaic virus</em></td>
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<tr>
<td>CP</td>
<td>Capsid protein</td>
</tr>
<tr>
<td>CPm</td>
<td>Minor capsid protein</td>
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<td>CTV</td>
<td><em>Citrus tristeza virus</em></td>
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<tr>
<td>Cu/ZnSOD</td>
<td>Copper and zinc cofactor/superoxide dismutase</td>
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<tr>
<td>FeSOD</td>
<td>Iron superoxide dismutase</td>
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<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
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<tr>
<td>HEL</td>
<td>Helicase</td>
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<tr>
<td>HLB</td>
<td>Huanglongbing disease (aka citrus greening disease)</td>
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<tr>
<td>HPV</td>
<td>Human papilloma virus</td>
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<td>HSP70h</td>
<td>Heat-shock proteins</td>
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<td>ISG15</td>
<td>Interferon-stimulated gene 15</td>
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<td>MnSOD</td>
<td>Manganese cofactor/superoxide dismutase</td>
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<td>MT</td>
<td>Methyltransferase</td>
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<td>NiSOD</td>
<td>Nickel cofactor/nickel superoxide dismutase</td>
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<td>PDS</td>
<td>Phytoene desaturase</td>
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<tr>
<td>PRO</td>
<td>Proteases</td>
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<tr>
<td>RdRp</td>
<td>RNA dependent RNA polymerase</td>
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<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
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<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
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<tr>
<td>SUMO</td>
<td>Small ubiquitin-like modifier</td>
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<td>TuMV</td>
<td><em>Turnip mosaic virus</em></td>
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Transmission is an essential part of the survival strategy of plant viruses. Little is known about the molecular process of vector transmission for many viruses. Here I examined the effect of the 5` end genes from different isolates of *Citrus tristeza virus* (CTV) for aphid vector transmission. Also, I examined the effect of silencing four plant-genes in citrus for CTV aphid vector transmission. CTV, which consists of at least 6 different strains, exhibits a variety of different phenotypic characteristics. These strains are VT, T30, T68, T36, T3 and RB, the nucleotide identities change between 80.5-92.4%. The 5`half of the CTV genome is more diverse than 3` end of the genome. To make matters more complex, in addition to these different strains, the rates of aphid transmission are different for different isolates of the strains. CTV encapsidation into long flexuous virions occurs via a complex mechanism involving at least four genes: CPm, CP, Hsp70 homologue and p61. The involvement of the Hsp70h and p61 proteins in aphid transmission has been reported. However, no work has been done to determine if there are replication-associated proteins involved in aphid transmission. I cloned the different 5`end genes from T68 and T30 isolates of CTV into our T36-based infectious clone, which is poorly transmitted by the aphid vector. Interestingly,
interchanging entire 5`half of genome produced a substantial increase in transmission by the aphid vector, from 1.5% transmission efficacy to 23.2% with the T68 substitution. Therefore, a further step was taken, and a new hybrid was constructed by swapping the p33 gene from T68 to T36 strain, followed by an aphid transmission experiment. This change increased the aphid transmission rate to 17.78%. This is the first report that the p33 gene is one of the important components of CTV transmission by aphid. Four proteins were studied through gene silencing by T36 vector; a miraculin-like protein-1, an iron superoxide dismutase, a ubiquitin-conjugating enzyme variant, and a cyclophilin. Three of the plant gene silencing CTV constructs, a miraculin-like protein-1, an iron superoxide dismutase, and a cyclophilin, resulted in increased aphid transmission rate of 5.94%, 24.44%, 7.41% respectively. The silencing of a ubiquitin-conjugating enzyme variant resulted 0% aphid transmission.
CHAPTER 1
LITERATURE REVIEW

Introduction

The citrus industry is an important part of Florida’s identity, as citrus has been commercially cultivated since the mid-1800s (https://www.floridacitrus.org/oj/facts). Citrus has great value as part of a nutritious and satisfying diet. The flavors of citrus are highly preferred all over the world, as citrus products are a rich source of vitamins, especially vitamin C, and are a source of nonstarchy polysaccharides, providing dietary fiber and minerals, as well as other carbohydrates (Economos and Clay, 1998).

Impact on Economy

The citrus industry generates roughly 76,000 full-time and part-time jobs. In addition to providing employment, the industry makes an important contribution to economic activity within Florida each year. The total impact of citrus on Florida’s economy is approximately $9 billion per year. The industry generates close to $1 billion in tax revenues for Florida, which helps to support schools, highways, and healthcare services (www.flcitrusmutual.com/citrus-101/citrusstatistics.aspx).

Florida citrus growers produce several types of citrus, including oranges, grapefruit, and specialized fruit such as Temple oranges, tangerines, and tangelos. Florida was used to produce 63 percent of U.S. citrus production (Florida Citrus Statistics 2012–2013 and 2016–2017 USDA, National Agricultural Statistics Service). Citrus is one of the major fruit crops in the world. Global orange production is forecasted to decrease in the 2017/2018 season. Unfavorable meteorological conditions led to a decrease in citrus yields in Brazil, the EU (European Union), and the United States (U.S.). U.S. production is projected to fall 23 percent to 3.6 million tons in Florida due to
pressure from citrus greening and partly due to effects from Hurricane Irma in September of 2017, which caused immature fruit to drop due to damaging high winds. (United States Department of Agriculture Foreign Agriculture Service. January 2018 https://apps.fas.usda.gov/psdonline/circulars/citrus.pdf). Drought has also adversely affected the crop in California. Global tangerine and mandarin production are forecast to be down slightly. Global grapefruit production is predicted to be up around 1 percent. However, overall, U.S. citrus production is projected to be down more than 20 percent. (United States Department of Agriculture Foreign Agriculture Service. January 2018 https://apps.fas.usda.gov/psdonline/circulars/citrus.pdf).

**History of Citrus tristeza virus**

Viral diseases can be devastating to commercial citrus growers. Citrus is cultivated worldwide between north and south latitudes up to 41 degrees (Bar-Joseph et al., 1989). The first reported epidemics of tree losses on sour orange (*Citrus aurantium* L) rootstock originated in South Africa in the early 1900s, which was later recognized as decline (Weber, 1943). Argentina experienced a major epidemic of *Citrus tristeza virus* (CTV) quick decline in 1930 that occurred in sweet orange trees on sour orange rootstock. The disease was named “tristeza” after the Spanish word for “sadness,” describing the general appearance of the affected trees. This decline occurred in Brazil as well in 1937 (Bar-Joseph et al., 1989); it also occurred in 1938 in sour lime in Africa (Hughes and Lister, 1949). CTV was first reported in California in 1939 (Fawcett and Wallace, 1946) and later in Florida (Grant, 1952) and became the most destructive viral pathogen of citrus (Moreno and Garnsey, 2010).
CTV can be spread quickly through insect or human vectors. It has killed more than one hundred million trees worldwide, mainly in South Africa, Argentina, Brazil, and the U.S.A.

In the 1930s in Argentina and Brazil, more than eighty million sweet orange trees grafted onto sour orange (*Citrus aurantium*) rootstock that were killed or declared unproductive due to decline (Bar-Joseph et. al., 1989; Moreno et al., 2008). Massive losses caused by CTV in Argentina (more than ten million trees), Brazil (more than six million trees) and the U.S. (more than three million trees) have been reported (Bar-Joseph et al., 1989). In Spain, more than 40 million trees, mainly sweet orange (*Citrus sinensis*) and mandarin (*Citrus reticulata*) grafted on sour orange, have declined progressively (Cambra et al., 2000). In addition, CTV may cause stem pitting in some citrus varieties, regardless of the rootstock used, which can be responsible for important losses of fruit quality and quantity (Moreno and Garnsey, 2010). The aphid vector, *Toxoptera citricida* (Kirkaldy), or Brown Citrus Aphid (BrCA), was the major factor for the spread of CTV, greatly increasing the severity and impact of the disease in Central America and the U.S. (Bar-Joseph et. al., 1989; Moreno et al., 2008).

Different CTV isolates cause a range of phenotypes, such as seedling yellows, quick decline in sweet orange grafted on sour orange, and stem pitting in grapefruit and sweet orange varieties (Garnsey, et al., 1987). Different CTV strains can infect the same host at the same time in nature, and no reliable method to obtain pure cultures of individual CTV strains exists (Tatineni et al., 2001).

**Taxonomy of Closteroviruses and Genetic Diversity of CTV**

*Citrus tristeza virus* (CTV) is a phloem-limited, aphid-borne virus, belonging to the family *Closteroviridae*, which contains about forty positive-strand RNA viruses
infecting higher plants (Agranovsky, 2016). The *Closteroviridae* viruses are typically flexuous filamentous rod-shaped virions that contain either mono-partite or bipartite positive-sense single-stranded RNA genomes (Dawson et al., 2013).

The recent International Committee on Taxonomy of Viruses (ICTV) reports that the family *Closteroviridae* is separated into four genera:

1. *Closterovirus*: thirteen species; examples of viruses in this group include *Beet yellows virus* (BYV) and *Citrus tristeza virus* (CTV);
2. *Crinivirus*: fourteen species, including *Lettuce infectious yellow virus* (LIYV);
3. *Ampelovirus*: nine species, including *Grapevine leafroll-associated virus 3* (GLRaV-3);
4. *Velarivirus*: seven species, including *Little cherry virus-1* (LChV-1) and *Grapevine leafroll-associated virus-7* (GLRaV-7).

In addition to these species, there are six unassigned virus species (Agranovsky, 2016; https://talk.ictvonline.org/taxonomy/).

The *Closteroviridae* are semipersistently transmitted plant viruses (Martelli et al., 2002. Flores et al., 2014). The *Closterovirus* genus (BYV and CTV) is transmitted by aphids; viruses of *Crinivirus* genus are transmitted by whiteflies, and viruses of *Ampelovirus* genus are transmitted by mealybugs (Ng and Falk, 2006). The vector for transmission of the *Velarivirus* genus has yet to be identified.

The genome of the *Closteroviridae* family of viruses is single-stranded (ss), positive-sense RNA and may be a single RNA (genera *Closterovirus* and *Ampelovirus*) or may be split between two or three components (genus *Crinivirus*). The size of the genome ranges between 15 and 19.3 kb. They have two conserved gene modules: I—the replicase associated gene contains one or two proteases (PRO), methyltransferase (MT), and helicase (HEL)-like domains and an RNA-dependent RNA polymerase
(RdRp) domain; and II—genes that have close homologues in all closteroviruses; a small membrane protein (p6), a homologue of heat-shock proteins (HSP70h), a large protein (p61), a capsid protein (CP), and a related minor capsid protein (CPm) (Agranovsky 1996; Dolja et al., 1994; Karasev et al., 1995; Karasev, 2000).

Based on the genome nucleotide sequencing classification of CTV, the genotype showed more than one strain of CTV. The study of Harper (2013) reported the complete genome phylogenetic analysis of CTV at least six present strains. A potential seventh strain (the recombinant isolate HA16-5) remains speculative until homologues are found (Melzer et al., 2010). Within the Closteroviridae, CTV is unique because it holds many distinct and characterized strains. These isolates contain a wide range of phenotypic combinations between their different hosts (Harper, 2013). The sequences of different CTV genotypes vary in unusual ways. It would be expected that the virus-strain sequences of all known isolates appear to be about similar at 90% or greater throughout the genomes. However, CTV isolates diverge progressively more toward the 5′-end, which was demonstrated in two CTV isolates (T36 and VT). The 5′-half of the genome may retain around 60% identity, while the 3′-half, which codes for the most virus genes, retains more than 90% of its identity (Mawassi et al., 1996; Hilf et al., 1999; Karasev and Bar-Joseph, 2010). At this time, sequencing groups have validated that CTV contains at least six different strains, exhibiting a variety of different phenotypic characteristics. These strains—VT, T30, T68, T36, T3, and RB—that represent most of the known isolates, and the nucleotide identities across the genome range between 80.5% (T36 and T68 lineages) to 92.4% (VT and T3 lineages) (Harper, 2013).
Host Range of CTV

CTV has limited host range naturally and experimentally. CTV infects plants of the Rutaceae family. Outside of the Rutaceae species, it also infects Passiflora gracilis (family Passifloraceae) (Muller et al., 1984). CTV infects commercially important citrus cultivars such as sweet orange, grapefruit, limes, lemons, and mandarins (Hilf, 2010), and some citrus relatives such as Aeglopsis chevalieri Swing., Afraegle paniculata (Schum.) Engl., and Pamburus missiones (Wt.) Swing., (Bar-Joseph et al., 1989).

The titer of viral infection varies among citrus species, hybrids, and citrus relatives. Poncirus trifoliata (L.) Raj is known to tolerate different CTV isolates, whereas some pomelos [C. grandis (L.) Osb.], kumquat (Fornunella crassifolia Swing.) were selectively tolerant to specific isolates (Gmitter et al., 1996; Mestre et al., 1997; Rai, 2006).

There exists slight understanding how of the CTV causes disease in some citrus but none in others. Three genes (p33, p18, and p13) of CTV are not required for the infection of most of its host but are needed in different combinations for infections of specific citrus species. These three genes were acquired to extend the host range by the virus (Dawson et al., 2013).

Diseases Caused by CTV

CTV is one of the most significant and damaging diseases the history of citrus industry as a viral pathogen. In nature, CTV exists as mixed-isolate population comprised of several virus strains, which often coexist within the same citrus trees (Grant and Higgins, 1957; Moreno et al., 1993; Rocha-Pena et al., 1995; Broadbent et al., 1996; Hilf et al., 1999; Kong et al., 2000; Niblett et al., 2000; Rubio et al., 2001; Roy and Brlansky, 2004; Hilf et al., 2005; Roy et al., 2005; Iglesias et al., 2008; Roy and
Brlansky 2009; Scott et al., 2013). As previously mentioned, CTV differs genetically as well as in symptoms in infected trees. CTV-infected tree symptoms vary due to several factors, such as CTV isolates, virus population structure, environmental conditions, and host species (Garnsey and Cambra, 1991; Garnsey et al., 2005). Syndromes caused by CTV isolates may visibly diverge based on virus population structure, host species, and environmental conditions (Figure 1-1; Figure 1-2; Figure 1-3) (Garnsey and Cambra, 1991; Garnsey et al., 2005). CTV can cause distinct syndromes:

**Decline.** It has been the most threatening disease caused by CTV; it results in the death of sweet orange on sour orange rootstock, mandarin, or grapefruit cultivars. Infected trees can die within a few weeks to a year because of viral effects on the phloem of the sour orange rootstock beneath the bud union. Often, there is a bulge that forms on top of the bud union, and honeycombing can be found on the inner portion of the bark flaps removed from the sour orange rootstock (Timmer et al., 2000; Bar-Joseph and Dawson, 2008; Dawson et al., 2013).

**CTV stem pitting (SP).** It is the abnormal development of the phloem, resulting in visible pits in the wood (Figure 1-2). Infected trees become stunted and tend to take on a bushy appearance. This does not result in the sudden death of the tree, but the productivity of the tree is reduced, which results in economic losses in sweet orange and grapefruit. Most of the CTV isolates cause stem pitting in Alemow (*Citrus macrophylla*) and reduce tree vigor (Timmer et al., 2000; Bar-Joseph and Dawson, 2008; Moreno and Garnsey 2010; Dawson et al., 2013).

**Seedling yellows (SY).** It is another result of CTV infection and can be temporary. When immature sour orange, grapefruit, or lemon trees become infected,
severe chlorosis and dwarfing occur only with some isolates, specifying that there are certain host factors involved in its expression (Figure 1-3).

Some CTV isolates show no symptoms in many citrus varieties. The T30 strain is known as the mild CTV isolate and lacks visible symptoms. Florida has two strains: T30 and T36, the decline strain (Dawson et al., 2013).

**The Genome of CTV**

CTV polar virions are composed of two capsid proteins and a single-stranded, positive-sense genomic RNA (gRNA) of approximately 20 kb, containing twelve open reading frames (ORFs) and two untranslated regions (UTRs) (Karasev et al., 1995). The 5'-'half of the genome encompasses two ORFs encoding proteins associated with viral replication that are expressed from the genomic RNA called the *replication gene blocks* and involve ORF 1a (encodes a 349 kDa polyprotein) and 1b (encodes a 54 kDa protein – RdRp domains) (Karasev et al., 1995). The ten 3'-proximal ORFs are expressed via 3'-coterminal subgenomic RNAs, and these include genes for the minor (CPm of 27kDa – p27) and major coat proteins (CP 25 kDa – p25) and proteins p33, p6, p65, p61, p18, p13, p20, and p23 (Karasev et al., 1995; Karasev and Hilf, 2010). The conserved *quintuple gene block* is related to virion assembly and functioning in the plant. This *quintuple gene block* consists of CPm, CP, p61, HSP70h, and p6 (Dolja et al., 2006). The CP, CPm, p61, and HSP70h are essential for virus particle assembly, and p6 is involved with the systemic invasion of host plant (Satyanarayana et al., 2000). Closterovirus-encoded HSP70 homologue and p61, in addition to both coat proteins, function in efficient virion assembly (Tatineni et al., 2008). HSP70h (65 kDa) is a homologue of the HSP70 plant heat-shock proteins (Karasev et al., 1995).
The p20 is a homologue of p21 of BYV and p33, p18, p13, and p23 with no homologue in the other closteroviruses (Dolja et al., 2006). CTV proteins p20, p23, and CP involve the suppression of RNA silencing (Lu et al., 2004). The p20 protein is the main component of CTV inclusion bodies (Gowda et al., 2000). The p33, p18, and p13 were shown to be CTV host-range determinants (Tatineni et al., 2011).

**Control and Management of CTV Diseases**

As mentioned previously, the interaction of CTV with citrus is very complicated and may produce a variety of diseases based on virus isolate and/or strain and scion cultivars in the area and on environmental conditions (Moreno and Garnsey, 2010). The method of controlling CTV is based on the frequency and severity of the isolates present in the field. The first strategy used for managing the diseases is exclusion or containment to prevent the introduction and spread of a new, severe isolate of the virus. Therefore, it is important to use an adequate sampling technique and a reliable detection method. Biological indexing on a sensitive indicator host had been used for years. Mexican lime and Alemow were mainly used for sensitive indicator hosts, because vein-clearing symptoms can be observed in these varieties (Figure 1-1) (Timmer et al., 2000; Bar-Joseph and Dawson, 2008; Dawson et al., 2013). The enzyme link immunosorbent assay (ELISA) was a breakthrough, developed for the detection of CTV coat protein and different monoclonal antibodies, added to the separation of some CTV strains (Permar et al., 1990; Vela et al., 1986). MCA13 is the monoclonal antibody widely used in Florida to distinguish between isolates causing decline and mild isolates (Permar et al. 1990).

The most effective management for CTV diseases is to accomplish CTV-free growth with use of clean budwood and prevent the new introduction of the isolates by
infected budwood or aphids. The first strategies include using certified budwood, controlling aphid populations, and following with routine inspections for effective disease management (Timmer et al., 2000). Australia, Brazil, and South Africa have been using cross-protection to control crop losses due to stem pitting and to maintain profitable commercial production (da Graça and van Vuuren, 2010).

Another effective management of CTV involves strategies to manage CTV quick decline through the use of CTV tolerant rootstock instead of sour orange, but control of stem-pitting (SP) poses additional problems. It has only identified one genetic source of resistance to CTV among Citrus species. However, mandarins are measured to be tolerant. Additionally, trifoliate orange (a sexually compatible genus related to Citrus) is a known source of resistance to create a tolerant hybrid. For example, hybrid rootstocks have been made with trifoliate orange (Poncirus trifoliata) and its hybrids Carrizo and Troyer citrange (sweet orange × P. trifoliata) and Swingle citrumelo (grapefruit × P. trifoliata). These rootstocks have genes that make them tolerant to CTV (Gmitter et al., 1996; Fang et al., 1998). Unfortunately, a new, resistance-breaking (RB) strain of CTV has been identified (Dawson and Mooney 2000; Harper et al., 2008; Harper et al., 2010). This strain of CTV was able to infect citrus plants grown on the trifoliate orange or its hybrids, causing mild to severe SP (Dawson and Mooney, 2000).

**Use of CTV as a Gene-Silencing Vector**

RNA interference (RNAi) is a highly specific gene silencing phenomenon triggered by dsRNA. Plants use RNAi to defend themselves against virus infection. This leads to the manipulation of the expression of endogenous genes.

Virus-induced gene silencing (VIGS) is an effective virus-vector technology tool for gene-function analysis in plants. VIGS technology also is a reverse genetic
technique that has been widely used to study the biological function of plant genes (Romero et al., 2011). After initial technology introduction in 1995, VIGS has been applied over the years in about thirty different species, including Arabidopsis thaliana, Nicotiana tabacum, and Solanum lycopersicon (Becker & Lange, 2010). It has been successfully applied in many species, although VIGS’ efficiency depends on the distribution and the movement of the virus in the plant (Romero et al., 2011).

Consequently, it is recommended to use an additional reporter gene, such as phytoene desaturase (PDS), to estimate the level of gene silencing (Senthil-Kumar et al., 2007).

VIGS development involves many steps (Becker & Lange, 2010). The initial step requires the insertion into the viral genome of sequences corresponding to the host gene to be silenced. After construction of the engineered virus, it needs to be carried to the host plant. The infected plant with the engineered virus initiates the synthesis of the viral dsRNA. Dicer-like enzymes cleave the dsRNA into short interfering RNAs. These RNAs are recognizable through RNA-Induced Silencing Complex (RISC) and are melted into single-stranded RNAs (ssRNAs), which are used afterward as template to target gene degradation.

Because most viruses have limited host range, many virus vectors have developed VIGS for plants such as Potato virus X (PVX), Tobacco mosaic virus (TMV), Tomato golden mosaic virus (TGMV), Tomato yellow leaf curl virus (TYLCV), and China virus satellite DNA (Igarashi et al., 2009). Because some of the reliable VIGS vectors can induce similar symptoms to those caused by the silenced gene (Igarashi et al., 2009), it is necessary to develop more VIGS vectors for additional plant species,
especially for woody crops. Development of more VIGS vectors will enhance plant-genomic studies (Burch-Smith et al., 2004).

A CTV strain T36-based vector has been developed for transient expression of foreign genes in citrus trees using a green fluorescent protein (GFP) as a reporter (Folimonova et al., 2007; Hajeri et al., 2014). It was assembled from a T36 strain of a CTV-based gene-silencing vector and demonstrated its silencing capabilities in silencing transgene GFP in *Nicotiana benthamiana* line 16c and citrus endogenous gene *PDS* in citrus.

The use of this CTV vector offers many advantages: it makes it possible to express foreign genes in citrus for many years (Dawson and Folimonova, 2013); it can be used for field applications to protect against diseases or to treat infected plants (Hajeri et al., 2014); and more than one foreign gene can be inserted into the CTV genome at the same time (Dawson and Folimonova, 2013; El-Mohtar and Dawson, 2014).

The genome manipulations of CTV showed that it could express foreign genes in the plant. A CTV-based expression vector is currently in use to develop a new research tool to identify antimicrobial genes with activity against the greening disease (Huanglongbing HLB); HLB is a destructive disease of citrus. Currently, no-resistant citrus or resistance-transgenic trees are available for growers. It is possible to use CTV-based vectors in the field to protect citrus trees until alternative treatments are available. This may be a new way to look at CTV to help in the battle with HLB (El-Mohtar and Dawson, 2014).
Figure 1-1. Citrus tristeza virus (CTV) leaf vein-clearing symptoms induced by CTV on A) and B) *Citrus macrophylla*, and C) *Nicotiana benthamiana*. Photo A September 5, 2012 Courtesy of Cecile Robertson. Photo B- and C March, 2018. Courtesy of Turksen Shilts.

Figure 1-2. Stem pitting of ‘Duncan’ grapefruit, A) stem with bark, B) stem with bark removed. Photo A and B January 23, 2006. Courtesy of Cecile Roberson.
Figure 1-3. Seedling yellows symptoms on A) ‘Duncan’ grapefruit; B) and C) sour orange seedling inoculated with CTV. Chlorosis is evident in C) and stunting is shown in (B). Photo A November 18, 2014, B and C September 19, 2003. Courtesy of Cecile Roberson.
CHAPTER 2
MATERIAL AND METHODS

Identification of the Plant Proteins Binding with CTV Particles

In order to identify citrus phloem proteins that interact or bind to CTV particles, protein overlay assay, also known as Far-Western, was used. In this experiment, bark tissue from citrus was removed and the proteins were extracted using phosphate buffer containing anti-proteases cocktail. Proteins were separated in monodimensional gel electrophoresis, followed by blotting onto a nitrocellulose membrane. After blocking the unoccupied areas with skimmed milk, the membrane was overlaid with purified CTV particles. After incubation, the membrane was washed and the immunodetection of CTV was carried out. The phloem tissue proteins that bound to CTV particles could be indirectly identified by the bands after the immunodetection of CTV. Four significant binding proteins were revealed and identified by LC-MS-MS as miraculin-like protein-1, iron superoxide dismutase, ubiquitin conjugation enzyme, and cyclophilin. This work was carried out by my advisor. In my work, I knocked down these four genes in citrus in order to study whether these genes were implicated in the transmission by the brown citrus aphid.

RNA Isolations

Total RNA was extracted using different methods for chimeric CTV hybrids and RNAi silencing hybrids.

**RNA isolation methods for infectious chimeric hybrids.** RNeasy Mini Kit (Qiagen) was used for total RNA extraction from 100 mg of bark tissues from previously infected and CTV ELISA-positive tested *C. macrophylla*. Samples were ground in liquid nitrogen and lysed under highly denaturing conditions by the lysis buffer provided in the
kit. After lysis, samples were centrifuged through a QIAshredder to remove insoluble material, and the supernatant was collected. Before samples were centrifuged, ethanol was added, which created conditions to promote the binding of RNA to the RNeasy membrane. Total RNA bound to the membrane, contaminants were washed away, and RNA was eluted in the 50 µl water. The protocol was followed for RT-PCR, described in this chapter.

RNA isolation methods for RNAi hybrids. Total RNA was extracted using two different methods. The first method was used for the cloning step, and the second method was used for real-time RT-qPCR. In the first method, total RNA was extracted from 100 mg of *C. macrophylla* tissue using a RNeasy Mini Kit (Qiagen), following the manufacturer’s recommendations. The second method total RNA extracted for the real time RT-qPCR by TRIzol® reagent (Invitrogen, Carlsbad, CA) as per the manufacturer’s instruction. A total of 100 mg citrus plant tissues was homogenized with a mortar and pestle by liquid nitrogen, and 1 mL of TRIzol reagent per 100 mg of tissue was added using and followed incubating the homogenized sample for 5 min at 15 to 30°C and adding 0.2 mL of chloroform. The samples were vortexed and incubated at room temperature for 2 min and centrifuged at 10,000 rpm for 15 min at 4°C. Following centrifugation, the colorless upper-aqueous phase was collected into a new centrifuge tube and was precipitated by adding 0.5 mL of isopropyl alcohol and was centrifuged again for 10 min at 10,000 rpm. The samples were air-dried, and the RNA pellet was resuspended in 500 µl water.

Reverse Transcriptase (RT-PCR)

AffinityScript One-Step RT-PCR Kit (Bio-Rad, Hercules, CA) was used as the complete system for single-tube RT-PCR. The complementary DNA (cDNA) was
amplified in the same tube by Herculase II fusion DNA polymerase. Complementary DNA synthesis and PCR took place during an uninterrupted thermal-cycling program. cDNA synthesis at 50°C for 50 min immediately followed by an RT heat-inactivation step at 94 °C and then 40 thermal cycles for PCR amplification. The amplification cycle consists of a denaturation step at 94°C for 30 s, a template primer annealing step at 58°C for 30 s, and an extension step if the target is bigger than 1 kb at 68°C for 1 minute per 1 kb. If it was a smaller size than 1 kb, then it was set at 72°C.

**Polymerase Chain Reaction (PCR)**

Vent DNA polymerase (New England Biolabs, Ipswich, MA) was used for PCR by diluting templates plasmids (1:50) or/and RT-PCR products following the manufacturer’s recommendations. PCR were performed in a S1000 TM Thermocycler (Bio-Rad, Hercules CA). Initially, there was a denaturing step at 94°C for 2 min, followed by 35 cycles of another denaturing at 94°C for 30 s, annealing 58°C for 30 s and 72°C for 1 minute per 1 kb, and a final extension step at 72°C for 5 min.

**Development of CTV Hybrids**

To confirm the effect of the replicase complex in aphid transmission, a series of hybrid clones was constructed by using the CTV-T36 full-length (GenBank accession #AY170468) infectious clone (Satyanarayana et al., 1999, 2003) plasmid as a cloning vector backbone. The nucleotides (nt) numbering is based on a full-length CTV isolate of T30 (GenBank accession #AF260651.1) (Albiach-Marti et al. 2000), the CTV isolate of T68 (GenBank accession #JQ965169.1) (Hilf, M.E., Harper, S.J. and Dawson, W.O.). A list of primers used in construction of CTV infection clones listed on Table 2.1. The first cloning strategy was finding the location of restriction enzymes unique and rare restriction sites within the genome of CTV strains. Consequently, I was able to exchange
fragments between different strains. Apal, Ascl, PspXI, Bsu36I, Xmal, and Pmel were used, which are describe in the drawing of the contract seen in Figure 2-1, and Figure 2-3. After completing the cloning of CTV chimeric sequences generated in this experiment, the results were confirmed true by the sequencing facility of Macrogen USA, MD.

Cloning from T68 Isolates of CTV

The T68 infectious hybrid clone was assembled through one-step reverse transcriptase (RT)-PCR amplification of three fragments of T68 isolate of CTV to the infectious clone of T36 by substitution.

The T68 infectious hybrid clone was constructed through the assembling the 5'-end of the T-68 strain of CTV, started from upstream of intergenic region of p6 trough to the 5'-end of being exchanged from I-Pmel to Xmal, II-Xmal to PspXI IV, and PspXI to Apal. The following cloning steps for building the T68 5'-end of the CTV infectious clone are shown in Figure 2-1.

The substitution of fragment assembling started from 11023 nt of CTV through the 5'-end:

Step I. Amplification of fragment consisting of nucleotides 8078 to 11023. Helicase, RdRp, and p33 genes from the T68 strain of CTV were amplified with forward primer-c2299, and reverse primer c2302 containing Xmal and Pmel restriction enzyme site, respectively, after digesting with Xmal and Pmel and fragment ligated into similarly digested based CTV-T36 infectious plasmid vector 35s-193 (35s193 clone vector is a modified T36 clone vector that contains VT 5'-end sequence until Ascl (182nt) site continue encodes L1 and L2 leader proteases of T30). This new was plasmid named 35s196 and was used for the following cloning steps.
Step II. The second phase of building an infectious clone of T68 was done by replacing the portion between parts of leader protease LP2 to helicase. Fragments comprising nucleotides 2026 to 8078 were amplified with forward primer-c2296 and reverse primer-c2300, including PspXI and XmaI restriction enzyme sites, respectively, followed digesting with same restriction enzyme and were ligated into similarly digested CTV-hybrid clone obtained from step-I. This new plasmid was labeled 35s198 PspXI restriction enzyme is unique in T68-11 it was also not the exact position in the T30-PspXI restriction enzyme site; this resulted in the duplication of LP2 in the portion of T68. This was represented by the green box in the drawing in Figure 2-1.

Step III. The last fragment of the T68-T36 hybrid cloning fragment was amplified with forward primer-c2267 and reverse primer-c2298, containing a flanking Apal restriction site position at the 35s (CaMV) promoter and a PspXI restriction enzyme site at nucleotide 2096. The amplified fragment and the hybrid clone from step II were digested with Apal and PspXI restrictions enzyme and ligated. This step finalized the cloning portion of T68 ORF1a and 1b, in addition to p33 until p6 was upstream of the intergenic region. This final vector was named 35s200, which was the infectious clone of the T68-T36 isolates hybrid for aphid transmission.

To confirm the role of the p33 protein in aphid transmission, another infectious chimeric hybrid was constructed using the T68 isolate of CTV (Figure 2-2). I generated this hybrid construct using 35s200 infectious cDNA clone of T68-T36 isolates hybrid for amplification of the p33 gene of T68 strain, with forward primer c2475 containing a Stul restriction enzyme site and reverse primer c2470 containing a PacI restriction enzyme site. The PCR product used for replacement between the Stul-PacI restriction enzyme
site in infectious CTV clone of 35s246 hybrid is the GFP replacement of the p33 gene (provided by El-Mohtar). This new infectious hybrid clone was named 35sT8, which included only the p33 protein from the T68 isolate of CTV.

**Cloning from T30 Isolates of CTV**

The T30 infectious hybrid clones were constructed through one step RT-PCR amplification of four fragments of T-30 isolate and exchanging fragments to infectious clone of T36-based vectors by swapping from the 3’-end toward the 5’-end.

**Step I.** Fragments comprised of nucleotides 8083 to 11828 were amplified with forward primer-c2459, and reverse primer c2281 containing Xmal and Pmel restriction enzymes side, respectively; product was digested with Pmel and Xmal and ligated into similarly digested-based CTV-T36 infectious plasmid vector. This portion includes nucleotides between HEL, RdRp, and the p33 portion from T30 isolates of CTV. The previously completed 35s201I, which is a chimeric hybrid of a VT-T36 infectious cDNA clone, was used as a vector. The vector 35s201I was digested with restriction enzymes Xmal and Pmel and replaced with the amplified and digested RT-PCR fragment. This new plasmid was named 35s231 and was used for following cloning step.

**Step II.** The fragment consisting of nucleotides 4431 to 8083 were amplified with forward primer-c2356 and reverse primer c2280, including Bsu36I and Xmal restriction enzyme sites, respectively. The amplified fragment was digested with same restriction enzyme and ligated into the similarly digested CTV-hybrid clone obtained from step I. This plasmid vector was named 35s232I.

**Step III.** DNA fragments covering nucleotides 182 to 4431 were amplified with forward primer-c2298 and reverse primer-c2357, including the Ascl and Bsu36I restriction enzyme sites, respectively. Similar steps taken in previous cloning of the
fragment was digested with the same restriction enzyme and ligated into the CTV-hybrid clone obtained from step II, which was digested with the same restriction enzymes. The newly created plasmid named 35s-233 was used in the final cloning step.

**Step IV.** The last fragment of hybrid clone was amplified containing the Ascl restriction enzyme site from 182nt with reverse primer c2267 to the Apal restriction enzyme site in 35s promoter of CaMV with forward primer c1882. The previous hybrid clone from step III was digested with Apal and Ascl restrictions enzyme and amplified-fragment ligated. At this point, cloning was finalized in the portion of T30 ORF1a and 1b in addition to p33 and the intergenic region upstream of p6. This final vector was named 35s230. The following cloning steps are shown in Figure 2-3.

**Cloning Strategy Used to Construct CTV Hybrids**

The first steps were to obtain the clone in the pGEM®-T Easy Vector Systems (Promega, USA) by extraction of the total RNA after the RT-PCR amplification. This system was convenient to clone PCR products generated by thermostable polymerases by adding a single deoxyadenosine, in a template-independent fashion, to the 3′-ends of the amplified fragments. There are also 3′-T overhangs at the insertion site; this is to provide a compatible overhang for PCR products and the system for a fast-ligation step.

Once it was determined that the clone was unable to replicate, the cloning strategy was changed. To eliminate possible mutation through subcloning, this step was removed, and the cloning process was started again, using between-restrictions enzyme sites by RT-PCR and exchanging the amplified fragment in the CTV T36-based cDNA infections’ clone vector. Independent clones were isolated at each cloning step, and these clones were used to assemble ten or more independent full-genomic cDNAs to limit the effects of potential errors within some components of population and
possible errors during RT-PCR and transforming the cDNA in *Escherichia coli*. T30 and T68 are found to be more highly toxic to *E. coli* than T36, and extremely toxic to *Agrobacterium tumefaciens*.

The existence of few unique restriction sites in the genome of T68 and T30 CTV made ligation of cDNAs into a full-genomic cDNA complex, requiring a sequence of five to six ligation and transformation steps and sequentially assembled by ligating ≈ 2-4-kb fragments into a modified 35s193, T36 based vector (Figures 2-1). CTV is usually a mixture of different isolates. Building an infectious clone from any isolate is much less complicated if the isolate only contains sequences from one strain. Amplified subclones from isolate populations also tend to have minor differences.

**Cloning RNAi Silencing Hybrids**

In this segment of the study, hybrid clones were constructed by previously used the CTV-T36 full-length (GenBank accession #AY170468) infectious clone (Satyanarayana et al., 1999, 2003) plasmid as a cloning vector backbone and in the binary vector pCAMBIA-1380 (Satyanarayana et al., 1999; Satyanarayana et al., 2001; Gowda et al., 2005; El-Mohtar and Dawson, 2014; Hajeri et.al. 2014).

The two plasmids were used that plasmid carrying GFP and without carrying GFP. The plasmid contained GFP between minor-coat protein (CPm) and coat protein (CP), and the plasmid was designated as 35sCTV-105 based on T36 isolate of CTV (Figure 2-4 and Figure 2-5). The plasmid with no GFP was named 35sCTV-PM. For both plasmid-cloning steps, the position, cloning primers, and digestion site were the same.

The 35sCTV-105 and 35sCTV-PM clones included duplicated 35S promoter of Cauliflower mosaic virus in the 5’-end, a ribozyme sequence of subterranean clover
mottle virus satellite RNA at the 3’-end. PacI and StuI restriction sites were engineered at the 5’- and 3’-ends, respectively, under CP subgenomic RNA controller element (CE) between ORF-p23 and 3’-untranslated regions.

In this study, the following items were of interest: citrus plant genes miraculin (Genbank accession #AB213395.1), iron superoxide dismutase (Genbank accession #EU883637.1e), ubiquitin conjugation enzyme (E2) (Genbank accession #GQ455410.1.), and cyclophilin (Genbank accessions #GQ853548.1) which were cloned into the 3’-end of CTV cDNA infectious clone for silencing each gene.

RNA extracted from CTV-infected greenhouse plants was used as a template for the amplification of miraculin, iron superoxide dismutase (SOD), ubiquitin conjugation enzyme, and cyclophilin. To clone a truncated fragment of genes, RNA was extracted with the Qiagen RNA Extraction Kit and amplified with the Affinity Script One-Step RT-PCR Kit (Agilent Technology). The resulting RT-PCR product was digested with restriction endonuclease PacI and was ligated into PacI and StuI digested 35sCTV-105/5sCTV-PM. All-new infectious cDNA clones of the CTV were named: RNAi#1 for miraculin-like protein1, RNAi#2 for iron superoxide dismutase, RNA#3 for ubiquitin conjugation enzyme, and RNAi#4 for cyclophilin.

The following primers were used in the amplification of genes for the miraculin gene with M1266 forward and M1267 reverse primers, for SOD gene M1268 forward and M1269 primers, ubiquitin conjugation enzyme M-1270 forward and M-1271 reverse primers, and cyclophilin M1272 forward and M1273 reverse primers (Table 2-2).

**Agrobacterium-Infiltration of CTV Constructs into N. benthamiana**

*Agrobacterium tumefaciens* strain EHA 105 was transformed with the binary plasmid carrying CTV. Agrobacterium-mediated inoculation of CTV construct into *N.*
*benthamiana* was performed as previously described, with minor modifications (Gowda et al., 2005). At the same time, with the help of CTV infection in *N. benthamiana*, additional silencing suppressors were transformed into EHA 105 with the heat-shock method by 5 min in the 37 °C water bath (Walkerpreach and Velten, 1994) and left to grow at 28 °C for 48 to 60 h on a 2xYT medium (Sambrook, et al., 1989) with an agar medium supplemented with antibiotics [Kanamycin (50 μg / mL) and Rifampicilin (50 μg / mL)]. Additional silencing suppressors were p22 of *Tomato chlorosis Crinivirus* (ToCV) virus (Cañizares et al., 2008); p19 of *Tomato bushy stunt virus* (Gowda et al., 2005); P1/HC-Pro of *Turnip mosaic virus* (Kasschau et al., 2003); and p24 of GLRaV-2 (Chiba et al., 2006). The suppressors were infiltrated at the same time to help establish the CTV infection (El-Mohtar and Dawson, 2014).

The ten individual colonies for each construct were grown overnight as seed cultures in 2xyt medium; with the same antibiotics added as above, 0.5 mL of the seed culture was used to inoculate 10 mL of same medium. The bacterial culture was centrifuged in a Sorvall-RC-5B centrifuge at 5,000 revolutions per minute (rpm) for 5 min and resuspended in 10 mM MgCl₂ and 10 mM 2(N-morpholino) ethane sulfonic acid (MES). The pellet was washed with 10 mM MgCl₂ and 10 mM MES and suspended in 10 mL induction medium at O.D. 600nm =0.5-1.0 (10 mM MgCl₂ and 10 mM MES containing 150 μM acetosyringone at a final concentration). This suspension was incubated at the room temperature (25 ± 3 °C) at least 4 h before injection into *N. benthamiana*. A needleless 1mL tuberculin syringe was used for the injection of the agrobacterium in the abaxial (lower) surface of *N. benthamiana* leaves.
Enzyme Linked Immunosorbent Assay (ELISA)

Triple Antibody Sandwich (TAS) ELISA used in this experiment was similar to the method developed by Garnsey and Cambra (1991). A new flush of citrus plants or young bark tissue was collected (around 0.25 g). Extracts were prepared by homogenizing the tissue in 5.0 mL of phosphate-buffered saline pH 7.8, containing 1% Tween-20 (PBS-Tween). Rabbit polyclonal antibody (1 μg / mL) was used for coating the ELISA plate. The Agdia A+B at 1:750K dilution was used as the detection antibody. Samples were replicated twice per plate. Reactions were read spectrophotometrically at 405 nm visible filtered on ELx800 Universal Microplate Plate Reader (Bio-Tek Instrument. Inc.) after 30 min and 60 min.

Virion Prep Extraction: CTV Virion Isolation and Inoculation to Citrus

After 8–9 weeks post infiltration, ELISA was performed as previously described, using antibodies specific to CTV virions from N. benthamiana leaves confirming the systemic infection of CTV (Garnsey and Cambra, 1991; Folimonova et al., 2010), followed by CTV virion isolations for bark-flap inoculation of C. macrophylla, as previously described (Gowda et al., 2005; Robertson et al., 2005), with the additional centrifugation step. The CTV virions have been concentrated through a sucrose step gradient centrifugation by Robertson et al. (2005). This protocol for citrus plant infection was described in Garnsey et al. (1977) and used with minor modification for bark-flap inoculation.

Beckman Ultracentrifuge (Beckman Coulter, Optima LE-80K-Ultracentrifuge) was used in the virion concentration of CTV hybrid constructs for the aphid transmission experiment. It was concentrated twice with SW28 and followed with SW41 rotors, while following the cushion-gradient protocol applied previously (Garnsey and Henderson,
Ten grams of systemically infected *N. benthamiana* tissue were collected and chopped in an ice-cold 50 mL of extraction buffer (50 mM Tris, with 10% sucrose containing 10mM DTT), followed by sorval centrifugation (10 min in 10,000 rpm in 4 °C) to pellet down to debris. The protocol continued by preparing the SW28 rotor centrifuge tube step gradient lay of 5 mL of extraction buffer containing 25% sucrose and 5 mL of 55% sucrose below 27 mL of chopped *N. benthamiana* tissue extract. Centrifugation was completed at 19,000 rpm for 16 h in SW28 Beckman centrifuge rotor. The lowest 1.5 mL of the sucrose step gradient was discarded; next, 2 mL of the gradient was saved to another tube, and the volume was brought up 11 mL to concentrate again with centrifugations with an SW41 rotor. The Beckman centrifuge tube layered 1 mL 70% sucrose in the bottom of the tube, continued with 1.15 min centrifugation with SW41 rotor. Finally, a hole was poked in the tip of the centrifuge tube and two 400 µL aliquots of CTV virion were collected. Purified CTV virion were used to infiltrate citrus plants by bark flap inoculation (Figure 2-6).

*C. macrophylla* seedlings of 12–18 months old and approximately 24” in height with stems of pencil thickness were selected for bark-flap inoculation (Robertson et al., 2005). Citrus plants were grown in greenhouse conditions, with temperatures varying between approximately 25–32 °C.

**Aphid Colonies**

The brown citrus aphid (*Toxoptera citricida*) (BrCA) was used for transmission of CTV. Healthy aphids were maintained on uninfected healthy *Choisya ternata* plants (Figure 2-7). *C. ternata* is known as Mexican orange, an evergreen shrub belonging to the Rutaceae family. *C. ternata* is known as Mexican orange because its flowers have a similar shape and scent to the closely related orange (Gledhill 2008). The BrCA was
reared on *C. ternata* under controlled conditions with a temperature of 22–24°C, and a photoperiod of 16/8 h (day/night) in insect-proof cages (61 x 61 x 92cm).

**Aphid Transmission Assays**

The apterous form of BrCA was used for the transmission studies. Groups of 50 to 70 aphids collected from the *Choisyra ternata* plants were fed on CTV graft-inoculated donor seedlings of Alemow (*Citrus macrophylla*) for a virus-acquisition access period of 24 h. Aphids were placed in groups of five to seven virus-free receptor-plants of Mexican lime (*Citrus aurantiifolia*) seedlings. The aphids were placed onto the seedlings for an inoculation access period of 24 h. After feeding, the plants were sprayed with the aphicide (Malathion). After the aphids were dead, plants were then taken to the insect-free greenhouse and were maintained at ambient temperatures for up to four months. Plants were checked for transmission of CTV by ELISA at eight weeks after inoculation. The transmission efficiency was defined as the percentage of infected plants.

**Real-Time RT-qPCR**

Applied Biosystems (AB) 7500 Real-rime PCR detection instruments were used for the quantification of CTV. Total RNA was extracted with TRIzol diluted (1:10), and the reaction mixture was prepared as recommended by the manufacturer. The previously described RT-qPCR method was used (Harper et al., 2014). For the amplification of both CTV and the reference genes, a Superscript™ III Platinum® One-Step qRT-PCR kit (Invitrogen) was used with 200 nM each of sense and antisense primers and 50 nM of CTV specific 6-FAM/BHQ-1 labeled Taqman probe with additional MgSO₄. The real-time RT-qPCR optimal cycling conditions were 50°C for 5 min, 94°C for 2 min, 40 cycles of 94°C for 10 s, and 60°C for 45 s. List of primers and probe used RT-qPCR in Table 2-2.
Plant Conditions

In this experiment, *Nicotiana benthamiana* (tobacco) and *Citrus macrophylla* (Alemow) were used. *N. benthamiana* plants were grown in a growth room (22–24 °C, 60% humidity and of 16/8 h daily light cycle). One-year old *C. macrophylla* seedlings roughly two feet tall, with a single stem, were used for inoculation of CTV. Citrus plants were maintained under greenhouse conditions at the Citrus Research and Education Center, Lake Alfred, Florida.

Plant Sources

All the plant sources for cloning came from the greenhouse collection of Dr. W. O. Dawson, UF CREC, Lake Alfred, Florida.

T68-1 was used in the experiments. T68 isolates of CTV were field isolated to find which aphid transmitted to separate CTLV from CTV isolates. T68-1 is an aphid-transmitted sub-isolate of T68 obtained with *A. gossypii*. It does not carry viroids. T68 is an exotic isolate of CTV isolated from an Ellendale mandarin illegally imported into Florida.

The plant containing T30 isolate is thought to be typical of the mild CTV isolates widely distributed throughout Florida, and the source was sequenced to define the T30 genotype. T30 was originally recovered from a Mexican lime plant. The T30 containing plant was a mixture of T30+FL278 and T30+VT isolates.

T36 is the “type” isolate for Florida decline strains of CTV and the most intensively studied and best characterized isolate of CTV. T36 source plants were obtained from the collection of Dr. Steve Garnsey, USDA, Orlando location.
Photography of Plants

Plant photos under white light were taken with a digital camera (Canon EOS Digital Rebel XTi 400D, Lake Success, New York). Microscopic observations were made with a Zeiss Stemi SV11 fluorescent stereoscope (Carl Zeiss Microscopy GmbH, Göttingen, Germany) equipped with an Omax A351404 camera by Omax-TopView3.7 software.
<table>
<thead>
<tr>
<th>Primers Name</th>
<th>Primers Sequence</th>
<th>Primers Descriptions</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1881</td>
<td>TTGGATGACATCCTCGTGGGATTCTCCACTCAGGAAA</td>
<td>Forward primer in T30 for LP1 (nts699-722)</td>
</tr>
<tr>
<td>C1882</td>
<td>CCCGCACTTGGGGAGAAACGTACG</td>
<td>Reverse primers in T30 for LP1 (nts 930-958)</td>
</tr>
<tr>
<td>C2267</td>
<td>GTTGGGCCCCGATCTCCTTGGCCCAAGATCAACATAATGGGACGA</td>
<td>Forward primer 5'-end of the 35s with Apal site</td>
</tr>
<tr>
<td>C2268</td>
<td>AATTCGATTCAAAATCCCGTGATCTCCGGAGCTCGAT</td>
<td>5'-end of T30 T30 exact 5'UTR</td>
</tr>
<tr>
<td>C2275</td>
<td>CTG AAC GTG GGA AGA TTG GGG ATT TCA GTT TTC CGA GT</td>
<td>Forward primer common to T30 and VT used nts 2391-2428</td>
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<tr>
<td>C2276</td>
<td>ACT CGG AAA ACT GAA ATC CCC AAT CTT CCC ACG TTC AG</td>
<td>Reverse primer common to T30 and VT used nts 2428-2391</td>
</tr>
<tr>
<td>C2277</td>
<td>TCC TAG TCA TCA CTC GAG TGC CGC TCG TGG GCA ACG TT</td>
<td>Forward primer common to VT701 and 703 upstream of PspXI nts 2350-2387</td>
</tr>
<tr>
<td>C2278</td>
<td>TCA GTT TTT CGC GAT TTT TGT ACG ATT CGC GTT A</td>
<td>Forward primer upstream of second PspXI in VT701 strain only</td>
</tr>
<tr>
<td>C2279</td>
<td>CCC GTC GCA CGT GAC ATA ACG TAC AAG AAG ATG ACC AA</td>
<td>Forward primer Common VT and T30 nts 7796-7833, Upstream of Xmal</td>
</tr>
<tr>
<td>C2280</td>
<td>AGA CTA TGC TCC GAA TTA GTG AAC GTC AAA TCT TT</td>
<td>Reverse primer Common to VT and T30 Nts 8170-8205 Downstream of Xmal</td>
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<tr>
<td>C2281</td>
<td>ATA GCC ACC GTC GAA AAC GTG GTA CCA AAG TCT A</td>
<td>Reverse primer Downstream of Pmel common to VT and T30 nts 12051-12017</td>
</tr>
<tr>
<td>Primers Name</td>
<td>Primers Sequence</td>
<td>Primers Descriptions</td>
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<tr>
<td>C2296</td>
<td>AAG GTA TAA TTC GAG GAA GTC CTT CTA TAC GCG T</td>
<td>Forward primer for T68 upstream pf PspXI nts 1931-1964</td>
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<tr>
<td>C2298</td>
<td>ACT CGG AGG GCC AGC CGA ACG ACG ACT AAC ACC G</td>
<td>Reverse primer for T68 downstream of the PspXI nts 2207-2174</td>
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<tr>
<td>C2300</td>
<td>ACA GGA TAC TTT AGT ACA GTA GTC GGA AAA GTA CTT C</td>
<td>Reverse primer for T68 downstream of XmaI nts 8285-8249</td>
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<tr>
<td>C2302</td>
<td>ACC AAA GTC TAG ACC CAG AAG CAC CAT ACC GCT</td>
<td>Reverse primer for T68 downstream of the PmeI nts 12023-1199</td>
</tr>
<tr>
<td>C2331</td>
<td>AGT TCC TGA GGT ACG ATT TCA CGT ATC TCT CTA CG</td>
<td>Reverse primer for FL202 point mutation to create Bsu36I same position T36 nts 4421-4387</td>
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<tr>
<td>C2332</td>
<td>GTA CCT CAG GAA CTC GTT TAA TAT AAG TTT CGC</td>
<td>Forward primer for FL202 point mutation to create Bsu36I same position T36 nts 4408-444</td>
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<tr>
<td>C2348</td>
<td>TGAATGCTAAGACTTTTGAATGGACTTGGAA</td>
<td>Forward primer in VT downstream of Bsu36I to check recombination in 35s214</td>
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<tr>
<td>C2352</td>
<td>ACG TAG GTG GTT GCC CAT TAT TTC ATT TAC GTA AGT TTC TGC TTC TAC CTT TGA</td>
<td>Forward primer 5’-end of intron with extension to CTV nts 3357-3386</td>
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<tr>
<td>C2353</td>
<td>TCA AAG GTA GAA GCA GAA ACT TAC GTA AAT GAA ATA ATG GGC AAC CAC CTA CGT</td>
<td>Reverse primer 5’-end of intron with extension to CTV nts 3357-3386</td>
</tr>
<tr>
<td>C2354</td>
<td>ATA TAT GAC CAA AAT TTG TTG ATG TGC AGG TGC GGG TCA TAC AGG AGT TCA CGT TTG CA</td>
<td>Forward primer 3’-end of intron with extension to CTV nts 3387-3416</td>
</tr>
<tr>
<td>Primers Name</td>
<td>Primers Sequence</td>
<td>Primers Descriptions</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>C2355</td>
<td>TGC AAA CGT GAA CTC CTG TAT GAC CCG CAC CTG CAC ATC AAC AAA TTT TGG TCA TAT AT</td>
<td>Reverse 3'-end of intron with extension to CTV 3387-3416</td>
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<tr>
<td>C2356</td>
<td>CATACCTCGAGGAACCTCGTTTAATATGAGTTTCGTAGCT</td>
<td>Forward primer of T30 to create a Bsu36I similar to T36</td>
</tr>
<tr>
<td>C2357</td>
<td>GTTCCTGAGGTATGATCTCACGCACCTCTCCACA</td>
<td>Reverse primer of T30 to create a Bsu36I with switch of T to C nts to match to T36</td>
</tr>
<tr>
<td>C2459</td>
<td>TTGCAGACGACGTTTGGTGCTTTGCCGGGTCCGAAAAAACGCGATTCGCT</td>
<td>Forward primer of T30 with creating of XmaI in the T30 nts 8061-8112</td>
</tr>
<tr>
<td>C2470</td>
<td>ACCTTAATTAATCATATAAATATGATGGCTATCAAAACCCTCAT</td>
<td>Reverse primer T68-P33 with Pacl site</td>
</tr>
<tr>
<td>C2475</td>
<td>AATAGGCCCTTGTGGCTCCTCGAGTGAAACCAAGATATTAGAAG</td>
<td>Forward primer of T68-p33 with Stul site starting 10855nt</td>
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Figure 2-1. Diagrammatic representation of 5'-end infectious hybrids cloning steps from T68 isolate into full-length infectious clone of CTV. 35s193 is CTV-T36 infectious plasmid vector which used for cloning vector in Step-I by digestion restriction enzymes Xmal and Pmel. Followed by ligation protocol using similarly digested RT-PCR product. 35s196 vector plasmid was used for cloning Step-II. RT-PCR amplified product and plasmid vector was digested with PspXI and Xmal restrictions enzyme and ligated. 35s198 plasmid vector was used for cloning Step-III. In this step of cloning PspXI and Apal restrictions enzymes was used and a similar protocol proceeded as described. 35s200 infectious clone hybrid of T68/T36 strains was used for aphid transmission experiments. The different colors represent different strains in the drawing; gray for T36 strain, yellow for T68 strain, blue for T30 strain, and purple for VT strain.
Figure 2-2. Diagrammatic representation of cloning steps of 35sT8 infectious hybrid. 35s200 hybrid used for amplification of p33 gene of T68. Hybrid 35s246 is an infectious cDNA clone of T36 strain of CTV which containing GFP replacement of p33 gene. The 35s246 hybrid and amplified fragment from 35s200 digested with restriction enzymes Stul and Pacl, and GFP swapped with amplified p33 gene. The new infectious hybrid clone is named 35sT8 and was used in aphid transmission experiment. The different colors represent different strains in the drawing; gray color for T36 strain, yellow color for T68 strain. Green color represents GFP.
Diagrammatic representation of 5'-end infectious hybrids cloning steps from T30 isolate into full-length infectious clone CTV. 35s201I CTV-T36/VT infectious plasmid vector which was used for cloning vector in Step-I by digestion restriction enzymes XmaI and PmeI. Followed by ligation protocol using similarly digested RT-PCR product. 35s231I vector plasmid was used for cloning Step-II. RT-PCR amplified product and plasmid vector digested with PspXI and Bsu36I restrictions enzyme and ligated. 35s232I plasmid vector was used for cloning Step-III. In this step of cloning Bsu36I and Ascl restrictions enzymes was used and a similar protocol proceeded as described. 35s233 plasmid vector used for final cloning Step-IV and included between Ascl and Apal restrictions enzymes. The same protocol followed from previous steps. 35s230 infectious clone hybrid of T30/T36 strains was used for aphid transmission experiments. The different colors represent different strains in the drawing; gray for T36 strain, blue for T30 strain, and purple for VT strain.
Table 2-2. List of primers used in building CTV-RNAi silencing vectors and primers used for real-time RT-qPCR amplification and quantification of CTV in this study (Harper 2013; Harper et al., 2014).

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer Sequencing 5'-3'</th>
<th>Primer Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>M-1266</td>
<td>TCAAAGGCCTTCCCTTCTTATTTGCTTTGA</td>
<td>Forward primer Miraculin-like protein. Gene Bank Accessions # AB213395.1</td>
</tr>
<tr>
<td>M-1267</td>
<td>AGGTTTAATTAATAGATCTTGGGACTTTGCTTG</td>
<td>Reverse Primer Miraculin-like protein Gene Bank Accessions #AB213395.1</td>
</tr>
<tr>
<td>M-1268</td>
<td>TCAAAGGCCTAAGCAACACAGGCTATATGGATAAA</td>
<td>Gen Forward primer 2-iron superoxide dismutase Gene Bank Accessions #EU883637.1e</td>
</tr>
<tr>
<td>M-1269</td>
<td>AGGTTTAATTAAGTAGTAAGCGTGCTCCCTAGCATCA</td>
<td>Reverse primer 2-iron superoxide dismutase Gene Bank Accessions # EU883637.1e</td>
</tr>
<tr>
<td>M-1270</td>
<td>AACAGGCGCTGATCTCAGCTACCTACATCAAAATC</td>
<td>Forward primer Ubiquitin conjugating enzyme Gene Bank Accessions # GQ455410.</td>
</tr>
<tr>
<td>M-1271</td>
<td>TCGATTTAATACGATGAAGCCATGGGAGATG</td>
<td>Reverse primer Ubiquitin conjugating enzyme Gene Bank Accessions #GQ455410.1</td>
</tr>
<tr>
<td>M-1272</td>
<td>TCAAAGGCCTTCTTCAATCAACCATCAAAAATAT</td>
<td>Forward primer Cyclophilin Gene Bank Accessions #GQ853548.1</td>
</tr>
<tr>
<td>M-1273</td>
<td>AGGTTTAATTAACGGCTTGATGATGCGGAGTCATTATCAT</td>
<td>Reverse primer Cyclophilin Gene Bank Accessions #GQ853548.1</td>
</tr>
<tr>
<td>T36-F</td>
<td>ACC TCG GAC AAG CGG GTG AAT T</td>
<td>T36 strain-specific forward primer Location nts 10817-10838</td>
</tr>
<tr>
<td>Primer name</td>
<td>Primer Sequencing 5'-3'</td>
<td>Primer Description</td>
</tr>
<tr>
<td>------------------</td>
<td>-----------------------------------------------</td>
<td>------------------------------------------------------------------------------------</td>
</tr>
</tbody>
</table>
| T36-R            | GCA AAC ATC TCG ACT CAA CTA CC                | T36 strain-specific reverse primer  
Location nts 10885-10907 |
| *T36-RT-Probe    | 6-FAM-AGCAACCGCTGATCGATTGATT-BHQ1             | T36 strain-specific probe  
Location nts 10839-10861 |
| *ACTB-F          | GTTGCCATTGGTTGTATTTGATAC                      | Forward primer for RT-qPCR  
ACTB reference gene |
| *ACTB-R          | CGTCGACTGCCATTCCAGAT                          | Reverse primer for RT-qPCR  
ACTB reference gene |
| *ACTB-Probe      | 6-FAM-TGGTCGATGATTTGTCGATTTCCA-BHQ1           | ACTB reference gene probe for RT-qPCR |
| *GAPDH-F         | TGCGGACCAAGGCTACTC                            | Forward primer for RT-qPCR  
GAPDH reference gene |
| *GAPDH-R         | TTGCAGCACCAGTGTGATG                          | Reverse primer for RT-qPCR  
GAPDH reference gene |
| *GAPDH-Probe     | 6-FAM-TGCTAGCCACCGTGACCTCAGG-BHQ1             | GAPDH reference gene probe for RT-qPCR |
Figure 2-4. Schematic diagram of CTV genome. 35sCTV is infectious cDNA clone of T36 strain of CTV which was used for cloning of plant gene silencing hybrids. It shows the plant genes miraculin, iron superoxide dismutase (FeSOD), ubiquitin-conjugation enzyme, and cyclophilin insertion sites between restriction enzyme PacI and Stul in the infectious clone.

Figure 2-5. Schematic diagram of CTV-GFP vector genome. 35s105 is infectious cDNA clone of T36 strain of CTV which was used for cloning of plant gene silencing hybrids. It shows the plant gene miraculin, iron superoxide dismutase (FeSOD), ubiquitin-conjugation enzyme, and cyclophilin insertion sites between restrictions enzyme PacI and Stul in the infectious clone.
Figure 2-6. Bark-flapping technique on *C. macrophylla* showing A) one-year-old seedling used for inoculation; B) bark flap inoculation; C) inoculated bark tissue. Photos July 15, 2009. Courtesy of Cecile Robertson.

Figure 2-7. Brown Citrus Aphid [*Toxoptera citricida* (Kirkaldy)] maintained on healthy *Choisya ternata* A) general view; B) close-captured on the plant. Photos March 2, 2018. Courtesy of Turksen Shilts.
CHAPTER 3
THE TRANSMISSION PROCESS OF THE PLANTS VIRUSES

Introduction

Viruses are obligatory parasites, and their survival depends on transmission from one host to the next host. Viral pathogens have developed sophisticated strategies to improve the success of transmission events. There are two major mechanisms of transmission: 1) mechanical, and 2) invertebrate vectors. The first type occurs via plant material, such as seed transmission, pollen transmission, vegetative propagation, and grafting. The second type of transmission by invertebrate vectors involves organisms such as insects, beetles, and nematodes. The most common insect vectors are aphids, leafhoppers, and whiteflies. Some viruses are transmitted solely by fungus (Hull, 2009; Matthews, 2004).

There are over 383 known species of animal vectors of plant viruses. Invertebrate vectors that transmit many viruses from plant to plant are members of the Insecta and Arachnida classes of Arthropoda, and the Dorylaimida order of the Nematoda. About 94% of these vectors are arthropods, and 6% are nematodes. From the arthropod vectors, 99% are insects, and from these insects, 55% are aphids. Aphids are of the insect family “Aphididae,” suborder “Homoptera,” and they feed by sucking on green plants. “Homoptera” is an extensively used generic term for several suborders of the Hemiptera (Nault, 1997; James and Keith, 2004; Hull, 2009; Harris, 2018). There is ongoing work regarding the aphid species classification; the most widely consulted list is that of Blackman and Eastop (2000), with revisions available on the web at http://aphid.speciesfile.org. According to Van Emden and Harrington (2017), there are over 5,000 species of Aphididae in the world. Around 450 species occur on crops
(Blackman and Eastop, 2000), but only about 100 species cause significant economic problems. Fourteen species of aphids are listed as the most serious agricultural pests (Van Emden and Harrington, 2017). At least 288 aphid species have been tested for the possibility as a vector, and 277 aphid species have been shown to be able to transmit at least one plant virus (Nault, 1997). It is known that the major vectors of the plant viruses are aphids and whiteflies because they are able to transmit more than 500 virus species (Fereres and Raccah, 2015).

Plant virus transmission is a complex phenomenon concerning specific interactions between the virus, the vector, and the host plant, combined with the effects of environmental conditions. To better understand the progression of transmission, it can be thought of as a continuum of three overlapping processes: (1) acquisition time/phase, when the vector starts feeding or probing in an infected plant and acquires enough virus for transmission; (2) latent time/period, which is the period after acquisition time and before the virus can be readily inoculated by the vector; (3) retention time/transmission phase, which is the length of the time during which the vector can transmit the virus to a healthy plant (Matthews, 2004; Ng and Zhou, 2015). For successful virus transmission, all three of these activities should be optimal.

Piercing-sucking insects’ feeding behavior is not directly observable as it occurs inside the plant tissue. Monitoring, quantification, and analysis of different insect-feeding behaviors, such as sampling, phloem-seeking, and phloem-feeding, are identifiable by electrical penetration graph (EPG) (Walker, 2000). EPG studies have given evidence regarding the feeding behaviors of different hemipterans. Aphids are the most important vector group because of the number of viruses transmitted, and the damage caused
these viruses in crops, with the result that aphids are the most-studied vectors. Aphid feeding behavior was studied using EPG techniques, which are widely used to study these behaviors (Tjallingii, 2006). Aphids move their stylets in between cells located in the phloem sieve elements and tend sampling probes into these cells, including epidermal cells, early in the process (Tjallingii and Bennett, 1993; Fereres, 2015). Whiteflies are obligate phloem feeders and feed on abaxial side of the leaf (Stafford et al., 2012). The stylet pathway to the phloem is twisting to intercellular probes, but whiteflies make far fewer intracellular punctures than aphids, and their stylets reach deep into the plant tissue (Johnson and Walker, 1999). Leafhopper stylets of some species are larger than the diameter of phloem sieve element, and they tend to penetrate plant tissues directly to the phloem, so that they may destroy cells along the way during feeding (Backus et al., 2005; Stafford and Walker, 2009).

For efficient transmission, the virus must be temporarily retained in the insect vector. Two virion retention sites have been recognized in the insect vector: maxillary stylets and the foregut region (a region comprising the cibarium, anterior pharynx, and posterior esophagus) (Ng and Zhou, 2015).

The transmission process of the plant viruses has two principal modes, based on interaction between viruses and their vectors. They are termed circulative, internally borne, or persistent if the virus circulates through the insect’s haemocoel. They are termed noncirculative, nonpersistent, or externally borne if the virus is carried on the cuticle lining of mouthparts or foregut. It is also known that the grouping is based on the sites of retention and the paths of movement through the vector (Matthews, 2004; Ng and Perry, 2004; Ng and Falk. 2006; Fereres and Raccah, 2015; Ng and Zhou, 2015).
The terminology first used by Nault (1997) describes both the retention and mechanisms of plant-virus transmission by Hemipteran vectors: (a) nonpersistently transmitted, stylet-borne; (b) semipersistently transmitted, foregut-borne; (c) persistently transmitted, circulative; and (d) persistently transmitted, propagative. These terms will be further discussed in the next section.

In addition to all these factors, there are rare environmental factors involving abiotic stress that influence the success of virus transmission. An experimental demonstration was conducted on the effect of a severe water deficit on the efficiency of aphid transmission of the *Cauliflower mosaic virus* (CaMV) or the *Turnip mosaic virus* (TuMV). Drought caused the vector-transmission rate to significantly increase for CaMV and TuMV (van Munster et al., 2017).

**Circulative Transmission of Plant Viruses**

If a virus is circulated internally within the vector, then it is called a persistent, internally borne, or circulative-transmitted virus. Persistently transmitted viruses can be circulative-nonpropagative or circulative-propagative. The persistent plant viruses require min to h for acquisition and can be retained for very long periods, frequently until the death of the vector (Uzest and Blanc, 2016).

The circulative nonpropagative viruses pass over the foregut into the anterior midgut, posterior midgut, and hindgut. The circulative propagative viruses first infect midgut cells and later infect other tissues (Hull, 2009). The transmission cycle of a persistent virus includes the following steps: (a) aphid inserts stylets to reach the phloem sieve elements; (b) there is ingestion from the infected host plant that spreads to the alimentary system of the vector; (c) the virus passes through the vector’s gut; (d) there is virus retention in internal tissues; (e) the virus passes to the salivary glands;
and then (f) the virus is transported by the saliva to plant phloem tissue (Gray et al., 2014).

Virus species in the families Luteoviridae, Nanoviridae, and Geminiviridae are transmitted in a circulative, nonpropagative manner. Luteoviruses are transmitted in a nonpropagative, circulative manner by aphids. The virus particles that are carried in the haemolymph need to cross the basal lamina of the accessory salivary gland in order to be ejected by the salivary secretions to the plant tissues. On the way to the exterior, virus particles must move across many barriers utilizing different proteins. Luteoviruses are dependent on small CP and the read-through protein for transmission (Fererea and Raccah, 2015; Whitheld et al., 2015).

Nanoviruses and begomoviruses are specifically retained in the principal salivary gland. Members of the Nanovirus genus include Faba bean necrotic yellow (FBYN) transmitted by aphids. CP protein is responsible for virus-aphid interactions, although there is one report that a helper component protein is required for the transmission (Franz et al., 1999; Gray et al., 2014).

Another circulative virus is the Tomato yellow leaf curl virus (TYLCV), which belongs to the genus Begomovirus of the family Geminiviridae. TYLCV is one of the well-known tomato-infecting begomoviruses and is transmitted by whitefly Bemisia tabaci (Kil et al., 2016; Gray, 2014). The virus is found in the hemolymph and salivary glands, and some evidence suggests that the virus may move directly from gut cells to salivary gland cells (Cicero and Brown, 2011).
A major group of circulative viruses and their insect vector is listed in Table 3-1, and principal features of the insect-transmitted viruses are listed in Table 3-2. Lists were modified from an article by Fereres and Raccah (2015).

**Noncirculative Plant Viruses**

The noncirculative transmitted viruses are termed externally borne, as they do not pass through the vector’s interior. Noncirculative transmitted viruses have two subclasses termed nonpersistent or semipersistent, depending on virion retention either in stylets or the foregut of the insect, respectively (Blanc et al., 2014; Ng and Walker, 2015). Nonpersistent viruses are acquired within seconds and are retained only a few minutes by their vectors. In addition, they can be inoculated right away after a very short period acquisition and retention time (Matthews 2004; Hull 2009; Uzest and Blanc 2016).

As previously mentioned, nonpersistent transmitted viruses were initially called stylet-borne transmitted viruses because it was thought that the virus was simply contaminating the outside of the stylet. Recent studies showed that stylet-borne viruses are retained at the distal tips of stylets and later released by salivary secretions as the insect salivates during feeding. Considering that food and salivary canals merge at the tip of aphids’ stylets, salivation may function to help bind virions and deliver into plant cells. For example, virions of CaMV are retained in the aphid’s stylet in an acrostyle formed by the merger of the food and salivary canal (Bak et al., 2013)

The semipersistent viruses acquired within minutes to hours and retained for several hours. Their transmission efficiency increases directly with the duration of acquisition and inoculation periods, and retention time changes from hours to days. Semipersistent transmitted viruses are known as foregut-borne viruses as well. As
foregut-borne viruses, virus particles are attached to the cuticle lining of the foregut (Matthews 2004; Hull 2009; Uzest and Blanc, 2016).

Many plant viruses are noncirculatively transmitted; for example, hundreds of plant viruses are found in the families Potyviridae and Cucumoviridae, and many well-studied insect-transmitted viruses are from the genera Alfamovirus, Caulimovirus, Cucumoviruses, and Criniviruses (Table 3-1).

Transmissibility and specificity between noncirculative viruses and their vectors depend on the coat protein of the virus and/or on additional virus-encoded helper proteins. Based on these known virus-vector interaction factors, transmission can be divided into two subgroups: 1) direct interaction between the virus capsid and the site of retention in the aphid; 2) indirect interaction involving a nonstructural virus-encoded protein—named a helper component, helper factor, or aphid transmission factor.

**Direct capsid protein interaction**

The Cucumber mosaic virus (CMV) is a member of the Bromoviridae family of virus and nonpersistently transmitted by aphids. CMV is a very good example of direct interaction between virus capsid proteins (CP) and their vector aphids. CMV-encoded CP involves direct links between capsid proteins to the binding site of the aphid vector, which is a key component of aphid transmission. The best evidence for this is that a purified virus can be transmitted directly by an aphid with an artificial feeding system, without any additional other proteins or factors (Gera et al., 1979; Chen and Francki, 1990; Ng and Falk, 2006). CMV strains can be transmitted with varying efficiency by different species of aphids. The three amino-acid mutations in the CP through the chimeric RNA3 cDNA construct affect the transmission of CMV by *Aphis gossypii.* Several studies showed that the transmissibility of CMV by *Myzus persicae* requires two
additional mutations in the CP (Perry et al., 1994; Perry et al., 1998; Perry, 2001). Charge alterations in the metal-ion binding (in the loop structure of βH-βI loop) can also alter aphid vector transmission (Liu et al., 2002).

Lettuce infectious yellows virus (LIYV) is transmitted semipersistently by the whitefly Bemisia tabaci (Tian et al., 1999). The LIYV minor-coat protein (CPm) plays a critical role in mediating virion retention to the vector’s foregut, and this was verified using a combination of biochemical and molecular approaches and by transmission assays (Chen et al., 2011). There was discussion associating the involvement of the CPm C-terminus in transmission, and that of the N terminal (approximately 71 amino acids) portion in interactions required for virion encapsidation (Ng and Zhou, 2015).

Indirect interaction involving helper components

The potyviruses and caulimoviruses have helper components (HC). In the case of potyvirus, HC interacts between a coat protein (CP) of the virion capsid and the binding site of the insect stylet (Blanc et al., 1997; Peng et al., 1998). The term “helper component” is used if transmissibility and specificity between noncirculative viruses and their vectors require the coat protein of the virus and virus-encoded helper proteins. For example, the Potato virus C (the C strain of Potato Y potyvirus, family Potyviridae) was not aphid transmissible. It became aphid transmissible, when PVY-C was coinfection with PVY in the plant (Watson 1960). Later, it was shown that if aphids were first given acquisition with PVY-infected plants (Potato Y potyvirus, an aphid-transmissible strain) and then to PVY-C-infected plants (nonaphid transmissible), PVY-C then became aphid transmissible (Kassanis and Govier, 1971).

This first description of the “helper component” (HC) was given by Govier and Kassanis (1974). It was shown that HC was a component from the sap of the PVY-
infected plants, not the PVY virion itself. Researchers were able to separate HC from the supernatant after ultracentrifugation was used to pellet PVY virions. Later, HC was mixed with purified virions and fed to aphids in vitro, and they became aphid transmissible again; perhaps the HC could be acquired first rather than being needed later via the acquisition of PVY virions. However, it was clearly demonstrated that HC was required for the aphid transmission of PVY. HC functions as the bridge between the potyvirus virion and the retention site in the aphid's maxillary stylets. This hypothesis was named the “bridge hypothesis” (Pirone and Perry 2002).

*Cauliflower mosaic virus* (CaMV) uses a helper-dependent transmission but requires two viral-encoded, helper proteins, P2 and P3 (Blanc et al., 2001). In the infected cells, P2 originates in the crystalline electrolucent-inclusion bodies, and P3 in association with virus particles. P2 is an 8-kD nonvirion protein that is believed to bind to the aphid, and P3 is a 15-kD protein that is anchored to CaMV virion. In-vitro overlays and aphid-transmission bioassays have confirmed that P2 binds to P3, but not to P4 (the major CaMV virion capsid protein), and P3 binds to P4 (Leh et al., 2001; Leh et al., 1999). These viral-encoded proteins P2 and P3 were later named “aphid transmission factors” (Ng and Zhou 2015).

Aphid Biology

Aphids are soft-bodied insects that have piercing, sucking mouthparts to feed on plant sap. They produce large amounts of “honeydew,” which is a sugary, liquid waste. Aphids are vectors of plant viruses, which makes them important in spreading diseases. Genus Toxoptera (Aphidini) are dark and are small- to medium-sized aphids. They are of East Asian origin, but two species are now widely distributed on citrus and various shrubs. The most important and the larger species occurring on citrus is the brown
citrus aphid (BrCA). BrCA has appear two different forms in the field: 1) apterae type (the adult wingless forms), and 2) alatae type (adult winged form). The adults are very shiny and black, and the nymphs are a dark reddish-brown. In the field, mixed colonies of two or more species are very common (Denmark 1990). The BrCA host range is limited to *Citrus* spp. and members of Rutaceae (Carver, 1978).

The BrCA life cycle is less complex than that of most aphids. BrCA is anholocyclic—without sexual generation. BrCA has no sexual cycle in the autumn, and thus, there are no males, no oviparae, and no eggs. In Japan, the aphid is shown as holocycle on citrus (Komazaki 1988); however, in the major citrus production areas like Florida, they are permanently anholocyclic and viviparous parthenogenetic females. BrCA increase very quickly under favorable conditions. The optimal temperature for BrCA growth is 25°C (Tang et al., 1999). Nymphs mature in six to eight days at temperatures of 20ºC or higher (Halbert and Brown, 1998).

BrCA population outbreaks occur every year in two peaks—one in the spring and another in the autumn (Michaud and Browning, 1999)—but they may occur in three peaks in Japan (Michaud 1998, Yokomi, 2009). No specific month can be determined for these outbreaks because they are dependent only on rainfall levels, which can change from year to year (Kindlmann et al., 2010).

The natural enemies of BrCA are predatory insects such as ladybeetles (Coleoptera: Coccinellidae) and hoverflies (Diptera: Syrphidae); these two species cause the greatest mortality (Michaud, 1998). It was calculated that a single aphid could produce a population of over 4,400 in three weeks in the absence of natural enemies (Komazaki, 1988).
Aphid Transmission of CTV

CTV is transmitted by aphids and can be introduced to plants artificially by grafting. It is mechanically transmissible by stem-slash inoculation of partially purified sap into the stems of sensitive indicators (Garnsey and Muller, 1988). Aphid transmission of CTV is an important area of study, as it is not well understood.

Four aphid species are considered to be the major vectors of CTV. The cotton or melon aphid *Aphis gossiypii* Glover, the spire aphid *Aphis spiraecola* (Patch), the black citrus aphid *Toxoptera aurantii* (Boyer de Fonscolombe), and the brown citrus aphid *Toxoptera citricida* (Kirkaldy) have been associated with the natural movement of CTV (Roistacher and Bar-Joseph, 1987; Brunt et al., 1990- Brunt, A., Moreno et al., 2008.) *T. citricida* is a very efficient vector of most CTV strains (Moreno et al., 2008). The brown citrus aphid (*T. citricida*), previously called *Aphid citricidus* or oriental citrus aphid, is almost globally distributed, and it was the first reported vector of CTV in Brazil (Lee and Rocha-Pena, 1992; Bar-Joseph et al., 1989). In addition to these four aphid vectors of CTV, another three less-efficient and lesser-known aphid vectors are *Myzus persicae* (Sulzer) (Varma et al., 1960), *Aphis craccivora* Koch, and *Uroleucon jaceae* (Linnaeus) (Varma et al., 1965).

The brown citrus aphid (BrCA) spread widely very quickly in the first year after it was discovered in Fort Lauderdale, Florida, in November of 1995 (Michaud, 1998). BrCA is believed to have originated from China. After 1900, it spread to Asia, Australia, New Zealand, Pacific Islands, South Africa, and South America. It moved through many countries of Central America, several islands in the Caribbean, and then finally to North America (Tsai, 2004).
According to Yokomi and Garnsey (1987), the experiment completed using A. gossypii in California found that CTV isolates could be grouped into three classes of transmissibility: low (around 0–4%); intermediate (5–16%); and high (17–62%).

CTV aphid transmission and epidemiology have been studied, but the molecular mechanisms of aphid transmission remain unspecified (Bar-Joseph et al., 1989). CTV is transmitted semipersistently by aphids with direct interaction between the virion coat proteins or nonvirion helper component, according to Ng and Falk (2006). It was reported the transmission of CTV from partially purified virion preparations (p25, p27, and p20) was proven to be one of the requirements for coat-protein-mediated transmission. CTV is not aphid transmissible as purified virions fed to aphids in vitro (Herron et al., 2006). It was suggested that particle purification should be used because of the absence of the required helper protein; however, this experiment has not been replicated (Ng and Falk, 2006). Sucrose gradient extraction is used for CTV virions extraction regularly and is successful for the experimental CTV inoculation of citrus (Folimonova et al., 2008; Tatineni, et al., 2008). However, it should be considered that the extraction of intact particles is difficult, and it has been shown that partially unencapsidated or fragmented CTV is less infectious (10⁴-10⁵ times) than intact virions (Satyanarayana, et al., 2001).

It is not completely understood which CTV genes or sequences are related to aphid transmission. It was demonstrated in vitro with fluorescently labeled virions that CTV binds specifically to the lining of the cibarium of the aphid. It was further determined that the CPm is involved in the vector interaction. Recently, Killiny et al., (2016) showed that the presence of two proteins, p61 and p65 (Hsp70h), reduces virion
binding in vitro, but proteases did not affect the binding of CTV virions. The research concluded that CTV binds to the sugar moieties of the cuticular surface of the aphid cibarium, and the binding involves the concerted activity of three virus-encoded proteins (Killiny et al., 2016).

CTV consists of at least six different strains that exhibit a variety of different phenotypic characteristics. As mentioned before, the strains are VT, T30, T68, T36, T3, and RB, and the nucleotide identity changes between 80.5–92.4% (Harper 2013). The 5’-end half of the genome is also more diverse than the 3’-end of the genome (Mawassi et al., 1996). To make matters more complex, in additional to these different strains, the rates of aphid transmission of the virus are different for each strain and are changed by the aphid species. Also, interaction between CTV genotypes affects the transmission of this virus, depending on the coinfection of different genotypes (Harper et al., 2018). The literature has many examples of complex field mixtures of two, three, or more genotypes of CTV by single aphid transmission that show that individual aphids transmit only some of the genotype diversity within the source plant (Ayllon et al., 1999; Brlansky et al., 2003; Broadbent et al., 1996; d’Urso et al., 2000; Roy and Brlansky, 2009; Sambade et al., 2007).

Infectious clones of CTV are available for the T36 strain (Satyanarayana et al., 1999). However, the T36 strain transmission rate is very low by BrCA (less than 2%) (Brlansky et al., 2003)—in some cases, as low as 0.5% (Harper et al., 2016)—while NZ-B18 (recombinant between T68 and VT) has more than 40% transmission (Harper et al., 2009). In the clonal T36 strain, the roles of various proteins in transmission have been studied but are still not fully understood. It was shown that CTV virion genes were
involved with aphid transmission. The two proteins mainly involved in aphid transmission were mapped to the Hsp70h and p61 (Harper et al., 2016). In addition to this finding of the interaction of viral protein (CPm and Hsp70) with the sugar moieties on the surface of aphid foregut, it was also found that the presence of two heat shock-like proteins, p61 and p65, reduces virion binding in vitro (Killiny et al., 2016).

**Objectives**

A long-term goal of research is to understand the biological and molecular characteristic relations between CTV, the host plant, and its aphid vector, which in turn will improve future use of the CTV-based vector in the field. The purpose of this project was study to the viral factor or factors required for CTV to transmit by aphid. As a continuation of previous studies, I first examined the proficiency of different CTV isolates for transmission by aphids, and I then explored for additional potential factors that play a role in this process. Next, I evaluated the possible mechanisms that a viral gene or genes could require for aphid transmission.

Therefore, the specific objectives of this research were as follows:

- To examine the viral factor(s) involved in CTV aphid transmission.
- To understand the molecular mechanism of the transmission process in vivo.

To check these concepts, I first built infectious clones with different 5’-proximal ends with diverse CTV strains and followed up by checking aphid transmissibility of the infectious clones in vivo. This helped to understand genetic sequence variations of strains affecting the CTV aphid-vector transmission.

- To examine the plant factors that could elicit-suppress aphid transmission by VIGS ability of CTV.

To check this concept, I cloned four plant genes to CTV for silencing, which were miraculin-like protein 1 gene from *Citrus jambhiri*, iron-superoxidase dismutase from
"Citrus maxima" gene, ubiquitin-conjugating enzyme and cyclophilin from "Citrus sinensis" genes.

The focus of this study is to understand CTV through the virus-vector relationship and to explore the influence of viral genetic diversity on virus transmission by an aphid vector and to examine the molecular mechanisms responsible for that effect.

As previously mentioned, CTV encapsidation into long, flexuous virions occur via a complex mechanism involving at least four genes: CPm, CP, Hsp70 homologue, and p61.

The molecular mechanism of transmissibility was investigated by examining the effect of exchanging genes from the highly aphid transmissible CTV isolate FS577 to the very low aphid transmissible T36 infectious clone. This T36-FS577 hybrid was included from p6 through the middle of p18 with p6, Hsp70, p61, CPm, and CP genes completely included. Other hybrids between T36 and FS577 strains were examined for their transmissibility by aphid. We exchanged the T36 Hsp70 and p61 genes with those from FS577 together and separately. Transmission of these hybrids with BrCA showed that hybrid substitution of both genes (Hsp70 and p61) increased transmission efficacy from 0.6 to 18% while substitution of p61 and Hsp70 (p65) individually raised transmission to 4% and 2% respectively. This result suggested that aphid transmission needs the action of many genes. These two proteins involved in aphid transmission were mapped to the Hsp70h and p61 (Harper et al., 2016). Hsp70 is a chaperone-like molecule; p61 and HSP70 both are required for virion assembly (Satyanarayana et al., 2004). CPm has been reported to be involved in vector interaction in other characterized closteroviruses (Tian et al., 1999; Chen et al., 2011). This led to the hypothesis that in CTV, in addition to CPm, other viral proteins possibly associate in
specific binding. In a recent study of the interaction between the CTV and its aphid vector, CPm (p27), p61, and p65 (HSP70) genes were confirmed as in-vitro binding proteins, and activity was localized in the cibarium of the aphid foregut (Killiny et al., 2016). However, no work has been done to determine whether there are replication-associated proteins involved in aphid transmission. It has been known that the 5'-end of the CTV genome is more diverse than the 3'-end of the genome. To make matters more remarkable, in addition to these different strains, the rates of aphid transmission are different for each strain.

As it was key to study the transmission process in the CTV-aphid system, I needed to obtain an infectious cDNA clone of CTV isolates. In constructing the clone, I included a sequence from the replication gene block, and p33 from the T30 and T68 isolates. Subsequently, I examined the viral determinants that affect rates of aphid transmission by examining chimeric viruses created by substitution of sequences from the highly transmitted T68 isolates into the poorly transmitted T36 isolate.

Results

System Used to Examine New CTV Construct at T36-Based Vector

Aphid transmission plays a vital part in the comprehension of one of the inadequately understood features of the CTV infection and the virus populations. CTV has a complex association with multiple isolates, genotypes, and sequence variants which can cohabit an individual host plant.

Here, I examined T30 and T68 isolates of CTV, which are genetically distinct and have varying transmissibility, by showing the effects of exchanging of sequences into an infectious clone developed from the poorly transmissible T36-CTV isolate.
The full-length cDNA clone of T36 strain of CTV and a hybrid T36 vector 35s193—a modified T36 clone vector that contains VT 5’-end sequence until Ascl (108nt) site that encodes L1 and L2 leader proteases of T30 (Figure 2-1)—was used for building new chimeric constructs to infect citrus plants (Satyanarayana et al., 1999; Satyanarayana et al., 2000; El-Mohtar unpublished data).

Additionally, I changed the growth medium for Agrobacterium-mediated transformation from LB medium to 2xYT growth medium. It has been shown that CTV-T36 infectious clone induced toxicity to Escherichia coli (Satyanarayana et al., 2003). The hybrids produced in this study were even more toxic to E. coli and Agrobacterium tumefaciens. 2xYT was an excellent growth medium to culture bacteria for over two weeks without depletion of essential elements of growth. This was first developed as nutritionally rich medium for the growth of recombinant strains of E. coli. Components such as tryptone and yeast extracts are found in large quantities within this medium. Also, sodium chloride was present in the medium and provides a suitable osmotic environment for the growing, replicating bacteria.

Previously, there were no successful methods used for directly inoculating citrus trees using RNA transcripts or Agrobacterium tumefaciens-mediated inoculation with cDNA constructs. It was shown that CTV could be amplified in N. benthamiana plants after agroinoculation (Gowda et al., 2005), and it was demonstrated that CTV can replicate and move systemically in N. benthamiana plants (Ambros et al., 2011). In this study, I have been successfully inoculating small C. macrophylla trees with these chimeric constructs of CTV by agroinfiltration into N. benthamiana plants and allowing to virions accumulate in the intact leaves. Subsequently, systemically infected N. benthamiana...
*benthamiana* leaves were collected, and CTV virions were concentrated by centrifugation through a sucrose step gradient (Garnsey et al., 1977; Robertson et al., 2005). After *C. macrophylla* trees were infected, inoculum from the first infected plant was used to graft new plants for the experiment.

Remarkably, these hybrid constructs functioned as infectious clones in the citrus host and were transmitted by aphids. The 5’-end genes of different CTV strains had differential effects on the aphid transmission of CTV.

**Aphid Transmission**

The result of differences in transmission efficacy of wild type CTV isolates T36, T30, and T68 were presented in Table 3-3. The aphid transmission experiments were completed using a total number of 215 plants for T68 with three replications, 127 plants for T30 with four replications, and 66 plants for T36 with three replications. Results showed that the transmission rate varied in these parent isolates: 25.77% to 80% for the T68 isolate, 0% to 15.3% T30, and 0 to 5% for the T36 isolate. The transmission rate averaged 44.18% (95 positive from 215 transmissions), 1.57% (2 positive from 127 transmissions), and 1.5% (1 positive from 66 transmissions) for T68, T30 and T36 isolates, respectively. Some of the T-36 aphid transmission plants died before ELISA testing was completed, which reduced the total number of plants tested for aphid transmission. Previously full-length infectious clones of T36 were tested to provide a baseline for constructed hybrid in this study. It was determined that the T36 cDNA infectious clone transmission rate with BrCA was 0.6% (Harper et al., 2016).

The T68 isolate was transmitted at significantly higher rate than T36 and T30 isolates. After proving the effects of the 5’-end terminus sequence variation on the transmissibility, CTV hybrids (35s200 and 35s230-37) were constructed. I developed
infectious cDNA clones of CTV hybrid which were construct and named as 35s200 (the hybrid contains 5’-end of T68 strain), 35s230-37 (a hybrid containing 5’-end of T30 strain), 35sT8 (a hybrid containing p33 from T68 and the rest of the T36 strain) were graft-propagated to five new additional C. macrophylla seedlings for aphid transmission to have enough donor plants.

The aphid transmissions were completed (Table 3-4) using a total number of 306 plants for 35s200 with six replications and 144 plants for 35s230-37 with four replications. The result was that the CTV hybrids transmitted at the rate of 9.09% to 31.82 % for the 35s200, and 0% for the 35s230-37. To examine the effects of CTV strain variation on transmissibility, I compared the transmission efficacy of T36 isolate of CTV with T68, T30, and T36 hybrids (Table 3-3). The results showed that T36 transmitted at the frequency of 1.5 % (1 positive from 66 transmission) whereas 35s200, a hybrid of T68/T36, was transmitted with a frequency of 23.20 % (71 positive from 306 transmission). The hybrid of T30/T36 (35s230-37) transmission rate was 0% (0 out of 144 plants). While these two infectious clonal hybrids differ by only the 5’-end terminus, these genetic differences explain aphid transmission differences. Most importantly, for the first time, it was shown that hybrid containing the 5’-end terminus of CTV-T68 genes were able to be transmitted by aphids, and T30 5’-end terminus genes are not aphid-transmissible.

Given the involvement of many genes in the replication and the virion assembly, it was necessary to ensure this change was not due to a difference in the viral titer. To prevent this bias, only plants with similar viral titers were used as donor plants for aphid transmission experiments. ELISA were completed in plants with infectious hybrids and
the plants with parent strains of hybrids. Table 3-5 shows that similar ELISA results were obtained for plants used in transmission experiments. Therefore, there was no direct correlation between the aphid transmission rate and virus titer.

I compared the transmission efficacy of CTV isolates T30, T68, and T36 with the infectious clone hybrids that were constructed in this experiment. The transmission rates for the parent isolates of T68 and T30 were higher than the infectious hybrid clone of these strains. The transmission rate of the isolate (wild type T68) was around 44.19% while the T68 hybrid clone had 23.20% transmission. However, T68 hybrid clone transmission rate was higher than the T36 isolates, which had a transmission rate of 1.5%. For the T30 5'-end replacement in the T36 infectious clone, the transmission rate changed slightly compared to the T36 and T30 isolates. The T30 isolates transmission level was 1.57%, while the T30 hybrid clone 35s230-37 had a 0% transmission rate.

From these results, I went a step further and developed an infectious cDNA clone of the T36 strain containing a single gene from the T68 strain, the p33 gene. The p33 was the first gene picked to exchange in the low aphid transmissible CTV clone because it is a nonconserved gene, and apparently was acquired to allow infection of certain varieties of citrus, while the virus can systemically infect other hosts (Tatineni et al., 2011). After cloning this gene, cDNA of CTV clone was named 35sT8. The aphid transmission experiment was completed with four replications to determine if this protein is required for aphid transmission of CTV. Results showed that the transmission rate increased to between 10% and 23% for CTV hybrids, compared to the parent isolate of T36. 35sT8 transmitted frequency of 17.78% (16 positives out of 90).
These outcomes suggest that successful aphid transmission requires the p33 gene’s and the other genes’ collaborative action and that p33 is possible that the helper component of CTV aphid transmission.

**Discussion**

The vector transmission of plant viruses is an important area of research, and interactions between the virus and its vector are very specific. Before the era of genome sequencing and bioinformatics analysis, basic vector transmission information was included as a criterion in plant-virus taxonomy. Today, known interactions between viruses and vectors are very specific and unique to each virus. However, the vector transmission has a common basis for plant viruses and transmission by their insect vectors. Plant viruses are retained in the vector until they are transmitted to their plant hosts. The virion binding sites are very precise and bind only to specific sites of the mouthparts or foregut in vectors (Uzest et al., 2010; Fernandez et al., 2010). The molecular mechanisms of CTV transmission by aphid species remains unknown even though there has been over 50 years of study (Moreno et al., 2008). It was determined that the 3’-end genes play a role in aphid transmission. It was indicated that p65 and p61 genes could be involved in aphid transmission (Harper et al., 2016). p65, a Hsp70 like molecular chaperone, and two proteins p61, p65, are both required for virion assembly (Satyanarayana et al., 2000). CPm has been reported to be involved in vector interaction in other characterized closteroviruses (Tian et. al., 1999; Chen et al., 2011). Therefore, the hypothesized that in CTV in addition to CPm, other viral proteins possibly associate in specific binding. The interaction between the CTV and aphid vector had also been studied, and CTV genes CPm (p27), p61, and p65 (HSP70) were confirmed as in-vitro binding activity localized to the top of aphid stylet (Killiny et al., 2016).
The foci of this study are to understand CTV through the virus-vector relationship, to explore the influence of viral genetic diversity on virus transmission by an aphid vector. Interestingly, the introduction of 5'-end genes from a T68 isolate of CTV to a T36 infectious cDNA clone produced the most significant increase in aphid transmission (23%). It had to be considered that creating chimeric hybrids might have effects on viruses, virion assembly, efficiency of the replication process, and movement of the CTV within the host.

These results presented other questions related to the virion assembly, cell-to-cell movement, and correlation with aphid transmission. Therefore, to determine whether differences in transmission efficacy of the hybrid’s replication had changed in viral load in the source plants, I tested the CTV titer in the donor plants used in the aphid transmission assays using ELISA to approximate virion titer, as previously described. The 5'-end of the genes did not appear to affect virion assembly in the graft-transmitted donor plants; all hybrids became infected with *C. macrophylla* systemically and accumulated similar virus titers.

It was recently discovered that three proteins of CTV—p65 (HSP70 homologue), p61, and p27 (CPm)—interact with its aphid vector (Killiny et al., 2016), and other studies followed about the effect polymorphism within these genes has on transmissibility (Harper et al., 2016). It has been shown that the replacement of the HSP70 and p61 genes from FS577 on the T36 infectious clone increased the transmission efficacy. Similarly, replacing the gene of the 3'-end of T36 (p6 until the middle of p18) infectious clones increased the transmission rate. However, this transmission rate increase in hybrids lower than the FS557 isolates (Harper et al.,
2016). This showed that the aphid transmission required another protein interaction during virus transmitting.

I observed an increase of virus transmission by *T. citricida* with T68 chimeric hybrid clone. Therefore, I focused to the fact that the p33 gene may have a role in aphid transmission. I proceeded to create a p33 hybrid of T36 cDNA infectious clone which contained only a p33 gene from the T68 strain. Aphid transmission experiments followed as soon as the new hybrid was constructed. Substituting the p33 gene from the T68 strain into the T36 strain increased the transmission rate from 1.5% to 17.8%.

These outcomes suggest that successful aphid transmission requires the p33 gene and the other 3'-end genes’ (such as CPm, or/and p65, or/and p61) collaborative action, and p33 is the key component of CTV aphid transmission. More questions arose and had to be addressed, such as whether there are other helper components. Further investigation is needed to determine how the other viral genes are involved.

The p33 protein is encoded only in the genomes of CTV isolates, is nonconserved, and is not known to have other significant homology with other proteins in other members of the *Closteroviridae* (Dawson et. al., 2013). Three genes p33, p18, and p13 are expendable for the systemic infection of the most *Citrus* spp. in the CTV genome. However, the p33 gene product performs a major role in extending the virus host range. The virus requires these genes for systemic infection of sour orange, lemon, calamondin, and grapefruit (Tatineni et al., 2011). In addition, p33 has been examined for the role in virus superinfection exclusion. Mutation within the p33 resulted in a loss of virus ability to exclude superinfection by the wild type CTV. However, these experiments were done only in the T36 isolate (Folimonova et al., 2010). New findings suggested
that p33 may be an effector (Sun and Folimonova, 2018). There has been no experiment completed to show its function in vector transmission of CTV until now.

The helper strategy has been reported both in RNA and DNA viruses. RNA viruses in the Potyviruses genera, and for DNA viruses in the Calimovirus genus showed they have HC and there is no report about sequence or protein structure similarity between these HC. In Potyvirus, the viral encoded helper component HC-Pro binds to sites on the coat protein, and in the Caulimovirus genera, two components are needed: p2, which binds to the aphid stylet, and p3, which bridges p2 and the viral coat protein (Drucker et al., 2002; Froissart et al., 2002; Ng and Falk, 2006; Pirone and Blanc, 1996; Uzest et al., 2007). It is essential to investigate CTV protein-protein interactions, and these could be tested in a similar manner as CaMV (Cauliflower mosaic virus) to determine binding location and efficacy.

Here, I hypothesized that CTV virions require many virally encoded components for successful aphid transmission and p33 is one of the key components of CTV. This posed some questions: Is the p33 protein bound to the aphid foregut, and if so, which protein binds to p33? Which of the viral proteins may be involved, and how does variation within the proteins involved moderate the differences in the aphid transmission phenotype? Furthermore, are the required motifs necessarily the same for transmission for the different aphids, A. gossypii and by T. citricida? The next research step was to determine if the p33 gene of CTV binds to aphid foregut.

Finally, does the p33 increase and/or move it faster to reach systemic infection in the host? In this study, ELISA results from donor plants did not answer this question. Overall, I found that CTV aphid transmission is more complex than the transmission of
other plant viruses. From this study, it has been discovered that the p33 protein is one of the key components of aphid transmission and required for the successful transmission of the CTV genome.
Table 3-1. Major group of viruses and insect vectors.

<table>
<thead>
<tr>
<th>Virus group</th>
<th>Mode</th>
<th>Persistence</th>
<th>Localization</th>
<th>Insect vector</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alfamovirus</td>
<td>NP</td>
<td>Few h</td>
<td>Stylets</td>
<td>Aphids</td>
</tr>
<tr>
<td>Carlavirus</td>
<td>NP</td>
<td>Few h</td>
<td>Stylets</td>
<td>Aphids or whiteflies</td>
</tr>
<tr>
<td>Caulimovirus</td>
<td>NP</td>
<td>Many h</td>
<td>Acrostyle</td>
<td>Aphids</td>
</tr>
<tr>
<td>Closterovirus</td>
<td>SP</td>
<td>Many h</td>
<td>Foregut</td>
<td>Aphids or mealybugs</td>
</tr>
<tr>
<td>Cucumovirus</td>
<td>NP</td>
<td>Few h</td>
<td>Stylets</td>
<td>Aphids</td>
</tr>
<tr>
<td>Enamovirus</td>
<td>P</td>
<td>Weeks</td>
<td>Salivary glands</td>
<td>Aphids</td>
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<tr>
<td>Fabavirus</td>
<td>NP</td>
<td>Few h</td>
<td>Stylets</td>
<td>Aphids</td>
</tr>
<tr>
<td>Luteovirus</td>
<td>P</td>
<td>Weeks</td>
<td>Salivary glands</td>
<td>Aphids</td>
</tr>
<tr>
<td>Macluravirus</td>
<td>NP</td>
<td>Few h</td>
<td>Unknown</td>
<td>Aphids</td>
</tr>
<tr>
<td>Nanovirus</td>
<td>P</td>
<td>Weeks</td>
<td>Salivary glands</td>
<td>Aphids</td>
</tr>
<tr>
<td>Potyvirus</td>
<td>NP</td>
<td>Few h</td>
<td>Stylets</td>
<td>Aphids</td>
</tr>
<tr>
<td>Sequivirus</td>
<td>SP</td>
<td>Few h</td>
<td>Foregut</td>
<td>Aphids</td>
</tr>
<tr>
<td>Begomovirus</td>
<td>P</td>
<td>Weeks</td>
<td>Salivary glands</td>
<td>Whiteflies</td>
</tr>
<tr>
<td>Crinivirus</td>
<td>SP</td>
<td>Days</td>
<td>Foregut/Cibarium</td>
<td>Whiteflies</td>
</tr>
<tr>
<td>Ipomovirus</td>
<td>SP</td>
<td>Days</td>
<td>Unknown</td>
<td>Whiteflies</td>
</tr>
<tr>
<td>Torradovirus</td>
<td>SP</td>
<td>Days</td>
<td>Stylets</td>
<td>Whiteflies</td>
</tr>
<tr>
<td>Badnavirus</td>
<td>SP</td>
<td>Days</td>
<td>Unknown</td>
<td>Mealybugs/leafhoppers</td>
</tr>
<tr>
<td>Curtovirus</td>
<td>P</td>
<td>Weeks</td>
<td>Unknown</td>
<td>Leafhoppers</td>
</tr>
<tr>
<td>Machlomovirus</td>
<td>SP</td>
<td>Many days</td>
<td>Unknown</td>
<td>Leafhoppers</td>
</tr>
<tr>
<td>Mastrevirus</td>
<td>P</td>
<td>Weeks</td>
<td>Unknown</td>
<td>Leafhoppers</td>
</tr>
<tr>
<td>Waikavirus</td>
<td>SP</td>
<td>Few days</td>
<td>Foregut</td>
<td>Leafhoppers</td>
</tr>
<tr>
<td>Comovirus</td>
<td>SP</td>
<td>Days</td>
<td>Unknown</td>
<td>Beetles</td>
</tr>
<tr>
<td>Sobemovirus</td>
<td>SP</td>
<td>Days</td>
<td>Unknown</td>
<td>Beetles</td>
</tr>
<tr>
<td>Tymovirus</td>
<td>SP</td>
<td>Days</td>
<td>Unknown</td>
<td>Beetles/Thrips</td>
</tr>
<tr>
<td>Ilarvirus</td>
<td>P</td>
<td>Days</td>
<td>Unknown</td>
<td></td>
</tr>
</tbody>
</table>

NP: nonpersistent; SP: semipersistent; and P: persistent.
Table 3-2. Principal features of the modes of virus transmission by insects

<table>
<thead>
<tr>
<th>Feature</th>
<th>External (noncirculative)</th>
<th>Internal-circulative Persistent*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duration of retention</td>
<td>Brief (few h)</td>
<td>Long (days to months)</td>
</tr>
<tr>
<td>Duration of acquisition and transmission</td>
<td>Brief</td>
<td>Intermedia (h)</td>
</tr>
<tr>
<td>Latent period</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Tissue where virus is acquired and inoculated</td>
<td>Epidermis and parenchyma</td>
<td>Epidermis, parenchyma and phloem</td>
</tr>
<tr>
<td>Preacquisition fasting</td>
<td>Increase transmission</td>
<td>No effects</td>
</tr>
<tr>
<td>Passage through</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Insect species specificity</td>
<td>Low</td>
<td>Intermediate</td>
</tr>
<tr>
<td>Sequential inoculation</td>
<td>Poor</td>
<td>Intermediate</td>
</tr>
</tbody>
</table>

*All circulative viruses except the Pea enation mosaic virus (PEMV) are transmitted in a persistent manner. PEMV is assisted by an umbravirus that allows the virus to invade tissues other than phloem, and the duration of the acquisition and inoculation periods is similar to viruses transmitted in a nonpersistent manner.

Table 3-3. Results from CTV isolate used for aphid transmission. Table shows number of replications, number of plants tested by ELISA, number of plants that became positive, and percentage of transmission.

<table>
<thead>
<tr>
<th>Parents isolates used for cloning</th>
<th>Number of replications</th>
<th>Total Plants Tested</th>
<th>Number of positive plants</th>
<th>Percentage of transmission</th>
</tr>
</thead>
<tbody>
<tr>
<td>T36</td>
<td>1</td>
<td>25</td>
<td>0</td>
<td>1.5 %</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>21</td>
<td>0</td>
<td>1 out of 66 plants</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>20</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>T68</td>
<td>1</td>
<td>97</td>
<td>25</td>
<td>44.18 %</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>93</td>
<td>49</td>
<td>95 out of 215 plants</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>25</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>T30</td>
<td>1</td>
<td>96</td>
<td>0</td>
<td>1.57 %</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>9</td>
<td>0</td>
<td>2 out of 127 plants</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>13</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>9</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>
Table 3-4. Results from CTV infectious clone hybrids used for aphid transmission. Table shows number of replications, number of plants tested for ELISA, number of plants that became positive, and percentage of transmission.

<table>
<thead>
<tr>
<th>Constructs Name</th>
<th>Number of replications</th>
<th>Total Plant Tested ELISA</th>
<th>Number of positive plants</th>
<th>Percentage of transmission</th>
</tr>
</thead>
<tbody>
<tr>
<td>35s200 T68/T36</td>
<td>1</td>
<td>96</td>
<td>17</td>
<td>23.20 %</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>22</td>
<td>7</td>
<td>71 positives out of 306</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>97</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>44</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>22</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>25</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>35s230-37 T30/T36</td>
<td>1</td>
<td>94</td>
<td>0</td>
<td>0 %</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>10</td>
<td>0</td>
<td>0 positive out of 144</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>20</td>
<td>0</td>
<td>144</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>20</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>35sT8 T36/p33T68</td>
<td>1</td>
<td>30</td>
<td>3</td>
<td>17.78 %</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>17</td>
<td>3</td>
<td>16 positives out of 90</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>17</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>26</td>
<td>6</td>
<td></td>
</tr>
</tbody>
</table>
Table 3-5. ELISA result from plants used for aphid transmission.

<table>
<thead>
<tr>
<th>Isolates/Hybrids</th>
<th>ELISA reading O.D. @ 405 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT-T30</td>
<td>3.02 ± 0.2</td>
</tr>
<tr>
<td>35s230-37</td>
<td>3.19 ± 0.2</td>
</tr>
<tr>
<td>T36</td>
<td>3.41 ± 0.05</td>
</tr>
<tr>
<td>WT-T68</td>
<td>3.50 ± 0.04</td>
</tr>
<tr>
<td>35s200</td>
<td>3.40 ± 0.1</td>
</tr>
<tr>
<td>35sT8</td>
<td>3.36 ± 0.02</td>
</tr>
</tbody>
</table>
CHAPTER 4
PLANT FACTORS THAT COULD ELICIT/SUPPRESS APHID TRANSMISSION OF CTV

Introduction

Insect transmissions of plant viruses have multilayered interactions between the plant-virus-insect vector. Viruses use sophisticated strategies to overcome obstacles, separating in the plants and barriers imposed by the plant cell wall. Virus uses the host cell and incorporate itself into the host cells genetic material induces it to benefit for viral replication, multiplication, encapsidation, and the spread of virus within the host. The insect vector plays a key role during the virus-infection process of many plants. Relations among virus-host and virus-insect have been studied on different levels involvement by pairing. The transmission depends on multidimensional virus-host-vector interactions. Therefore, I investigate insect vector transmission after manipulating the host through virus.

Citrus tristeza virus (CTV) has recently been utilized for expressing foreign genes and for gene silencing in citrus plants (El-Mohtar and Dawson, 2014; Hajeri et al., 2014). In slow-growing, higher plants such as crop trees, it takes a long time to produce transgenic plants to study the expression of a single gene and its function. However, using the CTV-based vector to silence the gene or to express a foreign gene is a much faster process. In this chapter, the powerful and promising role of using the CTV-based vector was used for studying the CTV-citrus host-aphid vector.

In order to understand molecular mechanisms underlying the transmission of CTV by aphids, I screened putative CTV-binding citrus plants’ proteins/molecules using the gene-silencing ability of CTV in citrus. The following proteins were studied: (1) a miraculin-like protein-1 [Citrus jambhiri] named RNAi#1; (2) an iron superoxide
dismutase, partial \([Citrus maxima](SOD)\) named RNAi\#2; (3) a ubiquitin-conjugating enzyme variant \([Citrus sinensis](E2)\) named RNAi\#3; and (4) a cyclophilin \([Citrus sinensis]\) named RNAi\#4.

**Miraculin**

Miraculin is a plant protein, which is known as glycoprotein extract, a sugar substitute. It binds to the sweet receptor on the tongue. It was first purified from \(Richadella dulcifera\) red-berry extracts (Brouwer et al. 1968; Kurihara and Beidler 1968; Shintaro et al., 2006).

Miraculin has a homodimer protein contain two glycosylated polypeptides that are cross-linked by a disulfide bond. There have been studies on the function and expression of miraculin in host cells with the ability to glycosylate proteins—for example, tomato, lettuce, and the microbe *Aspergillus oryzae*—but was not found in *Escherichia coli* (Matsuyama et. al.2009)

The study showed that miraculin continuously expresses in miracle fruit and transgenic tomato. The miraculin–GFP fusion protein has been found to accumulate in the intercellular spaces of tomato epidermal cells. It has been indicated that miraculin was secreted from the roots of transgenic tomato (Hirai et al. 2010).

**Discovery of Miraculin**

The miraculin was first purified from the fruit extract of \(*Synsepalum dulcificum*\) (Synonym \(*Richadella dulcifica*)\), also known as “miracle fruit,” in 1968. \(*Richadella dulcifica*\), a native shrub of West Africa, has the unusual property of modifying a sour taste into a sweet one (Brouwer et al. 1968; Kurihara and Beidler 1968).

Miraculin is not sweet but is a protein-triggered transformation of the structures of taste receptors on the cells of the tongue. Miraculin binds to the sweet receptor on the
tongue. Acidic foods that usually sour it would be sensed as sweet after the taste receptors interact with miraculin. This effect could last up to an hour (Koizumi et. al., 2011; Sanematsu et al., 2016).

**Structure and Function of Miraculin**

Reports have shown that the *Murraya koenigii* seed has a protein with trypsin inhibitory activity. A later study determined it to be a miraculin-like protein that provides a crystal structure. The protein consists of 190 amino-acid residues with seven cysteines arranged in three disulfide bridges. The study also revealed an amino-acid sequence association between a miraculin-like protein, a soybean Kunitz superfamily member. The major differences in the sequence were seen at primary and secondary specificity sites (Gahloth et al., 2010). Both miraculin and miraculin-like proteins have shown similar sequences to a soybean Kunitz family trypsin inhibitor, known as STI. The Kunitz-type STI family has proteins found in legume seeds. Chymotrypsin, trypsin, cathepsin D, and subtilisin inhibitors are from Kunitz-type proteinase inhibitors, which are part of the defense mechanisms of the potato plant against insect and phytopathogen attacks (Walsh and Twitchell, 1991; Suh et al.1991; Strukelj et al.1992; Ishikawa et al. 1994; Tatyana A.V., et al. 1998).

Kunitz-type STIs are found in rough coffee (Mondego et al. 2011), lemon (Tsukuda et al. 2006), and tomato (Brenner et al. 1998). These are miraculin-like proteins, which are reported to function as protectors in the plant against pests, pathogens, and environmental stress. Miraculin is thought to have evolved as a taste-modifying protein because it lost trypsin inhibitory activity during its functional evolution (Takai et al. 2013).
The function of miraculin protein and the localization of this protein in the plant cell showed that it is stimulated as a response to being wounded or to a microbe attack. *Citrus jambhiri* Lush has two miraculin-like proteins, RlemMLP1 and RlemMLP2. They showed accumulation expression due to a wound or microbe attack (Shintaro T. et al. 2006). RlemMLP2 also indicated antifungal activity by inhibiting spore germination and germination-tube growth of *A. citri* (Isshiki et al. 2001; Akimitsu et al. 2003). These miraculin-function studies suggest their roles in plant defense. RlemMLPs appear to be the first line of defense against pathogen attack.

Studies associated with Huanglongbing showed that miraculin and/or miraculin-like protein activity is higher in the infected citrus plants causing a bitter taste in the juice (Nwugo et al. 2016; Rawat N. et al. 2015; Ma, 2014 et al. 2014; Nwugo C.C. et al. 2013; Agustí. et al., 2008; Talon and Gmitter 2008). The researchers also identified R-specific probe sets, which encoded leucine-rich repeat proteins, chitinase, constitutive-disease resistance (CDR), miraculins, and lectins (Rawat et al., 2015). During the CTV infection in the ‘Westin’ sweet orange variety, miraculin-like protein differently expressed proteins among infected and noninfected samples that were identified through the mass spectrometry (ms/ms) (Dória et al., 2015).

Iron Superoxide Dismutase (FeSOD)

**Discovery of SOD**

Brewer (1967) identified a protein that later became known as superoxide dismutase. In 1968, two researchers from Duke University, Irwin Fridovich and Joe McCord, discovered the enzymatic activity of superoxide dismutase. The term “superoxide dismutases” (SODs) originated from the reaction between hydrogen radicals and iron to oxidize a wide variety of organic substrates. It is also known as
Haber-Weiss reaction (Winterbourn C.C. 1995). In 1973, researchers found iron SOD in *Escherichia coli* intracellular space, known as sodB (Yost and Fridovich, 1973)

**Structure and Function of SOD**

Iron SOD presents two diverse groups. The first group is homodimer-formed, which is comprised of two duplicated 20 kDa subunit proteins and 1- to 2-gram atoms of iron in the active center. This group FeSOD has been isolated from bacteria *Escherichia coli* (Yost and Fridovich, 1973) and the plant species, *Ginkgo biloba*, *Brassica calpestris*, and *Nuphar luteum* (Salin and Bridges, 1980). The second group of FeSOD is a tetramer of four equal subunits, which is mostly in higher plants with 2- to 4-gram atoms of iron in the active center. It was also isolated from prokaryotes (Alscher et. al., 2002)—for example, *Thermoplasma acisophilum* (Searcy and Searcy, 1981) and *Methanobacterium bryantii* (Kirby et al., 1981).

SOD is very effective enzyme to neutralize superoxide. SOD enzymes could be adding or removing an electron from the superoxide molecules because superoxide (O$_2^-$) (the oxygen molecule) is toxic to organisms. This takes the superoxide into one of two less-damaging types. SOD has been proposed as important for plant-stress tolerance (Alscher et al., 2002).

Most living organisms in the presence of oxygen contain isoforms of the superoxide-scavenging enzyme superoxide dismutase, or SOD. It is thought that high levels of iron trigger oxidative stress and are mostly reactive through iron-catalyzed Fenton chemistry. Some diseases have been associated with iron overload, causing severe oxidative damage. SODs are the detoxification of superoxide (McCord, 2002). Bacteria defend themselves against oxidative bursts by activating detoxifying enzymes
through the iron or manganese SOD pathway. This is actively seen in the human bacterial pathogen *Staphylococcus aureus*, which causes disease (Garcia et al., 2017).

Under stressed conditions, reactive oxygen species (ROS) increase as plants respond. The SODs are found in a cell as the first line of defense against ROS (Alschcher et al., 2002). SODs shield against superoxide toxicity that make them important antioxidant enzymes. There are four groups of superoxide dismutase that are characterized as copper (Cu/ZnSOD: copper and zinc cofactor), manganese (MnSOD: manganese cofactor), iron (FeSOD: iron cofactor), or nickel (NiSOD: nickel cofactor) (Abreu and Cabelli, 2010; Valeria et al., 2006; Perry et al., 2010). NiSOD is not found in plants (Bowler et al., 1994; Kliebenstein et al., 1998; Simon et al., 2005). All these SODs are in different parts of the cell. Fe SODs are found in the chloroplast (Alschcher et al., 2002).

The eukaryotes, bacteria, and archaea are all of the iron/manganese family of SODs. This group of SODs is generally dependent on either Mn or Fe for catalytic activity (Garcia, 2017). Superoxide dismutase is made of proteins in the plant chloroplast. These highly conserved proteins are iron-superoxide dismutase and copper/zinc-superoxide dismutase. Plants depend on them for the elimination of superoxide formed through photosynthetic electron transport and function in reactive oxygen groups' metabolism (Pilon et al., 2010).

The studies showed that in plants, FeSOD is active in the plastids (Bowler et al.1994; Kliebenstein, 1998), and studies propose the possibility of some FeSOD isoforms in cytosolic locations (Myouga et. al., 2008: Armbruster et al., 2009). Regulation and expression of FeSOD is related to the stage of plants. Major FeSOD
(FSD1) was expressed higher in younger plants, and it reduced the beginning of the flowering stages of the plants (Pilon et al., 2010).

It has been reported that there are no visual phenotype changes from the loss of major FeSOD (FSD1) and the cytosolic and plastid Cu/ZnSODs activity in Arabidopsis (Cohu et al., 2009). However, when the mutations were localized in chloroplast FeSOD isomers FSD2 and FSD3 superoxide, some phenotypes were observed (Myouga et al., 2008).

It has been reported that FeSOD protected the plants from winter freezes. Research showed that FeSOD activity showed increases in the winter survival of transgenic alfalfa plants (McKersie et al., 2000).

**Ubiquitin**

Ubiquitin is a small protein (76 amino acids) that is highly conserved among all eukaryotic organisms. Ubiquitination is involved in the regulation of many signaling pathways and plays an important role in protein homeostasis (Glickman and Ciechanover, 2002). Ubiquitin is involved in regulating many pathways, such as protein transferrin, protein degradation, DNA repair, apoptosis, virus budding and receptor endocytosis, transcription, cell-cycle control and cell signaling, hormone signaling, and plant immunity (Pickart, 2004; Mukhopadhyay and Riezman, 2007; Ryu et al., 2010; Lee et al., 2009; Lee et al., 2011; Trujillo et al., 2010). Ubiquitin helps to control gene expression and the processes of other proteins. This occurs by a process known as ubiquitination (ubiquitylation). In this process, a ubiquitin molecule binds to a substrate protein, altering its function. The ubiquitin gene produces a precursor molecule called polyubiquitin, which contains several identical ubiquitin repeats and two N-terminal ubiquitin moieties fused to ribosomal polypeptides. The C-termini of the precursor
molecules are cleaved by specific endopeptidases to release the mature 76 amino-acid proteins with a typical C-terminal di-Gly motif. Ubiquitination necessitates the sequential action of three different enzymes: E1, E2, and E3 (Randow and Lehner, 2009).

**Discovery of Ubiquitin and Functions**

Ubiquitin was isolated in 1975 from bovine thymus glands by a group of researchers and was later found in many different tissues (Goldstein et al., 1975). Using the reticulocyte lysate system, a scientific breakthrough was made by researchers in the late 1970s and 1980s, with the discovery of the ATP-dependent, ubiquitin-mediated protein-degradation system. The research performed from 1981 through 1983 confirmed the multistep ubiquitin-tagging hypothesis by isolating and characterizing three separate enzyme activities, E1, E2, and E3. The first recognized and purified ubiquitin-triggering activity involved E1 (Ciechanover et al., 1981). The discovery of ubiquitin-mediated proteolysis ended with the identification of the ubiquitin-conjugating enzyme E2 and the ubiquitin protein ligase E3 by the covalent affinity chromatography method. Next came the recognition the ubiquitin-activating enzyme E1 could not produce ubiquitin-protein conjugates by itself (Haas et al., 1981; Ciechanover et al., 1982; Hershko et al., 1983). Later studies revealed many ubiquitin-like proteins, such as small ubiquitin-like modifier (SUMO), autophagy 8 (ATG8), interferon-stimulated gene 15 (ISG15), and neural precursor cell expressed, developmentally downregulated 8 (NEDD8) (Kerscher et al., 2006).

Many human diseases are related to defects in proteolysis, including a variety of cancers. For example, human papilloma virus (HPV) infection often leads to human uterine and cervical cancers and is linked to ubiquitin-mediated proteolysis. The HPV tries to avoid p53 (a tumor-suppressor gene) by activating another E3 enzyme.
Eventually, an infected cell can no longer repair DNA damage in a normal way or go into apoptosis; finally, mutations accumulate, and a cancer may be formed (Scheffner et al., 1993). It was discovered that the ubiquitination of plasma-membrane proteins has important targeting functions in endocytosis and secretion (Hicke and Riezman, 1996). These breakthrough studies on ubiquitination showed the possibility of controlling the cell cycle for changing certain biological processes.

Ubiquitination may affect protein activity, location, or interactions with other proteins. Ubiquitin can also modify DNA transcription and repair, cell death (apoptosis), immune and inflammatory response, cell division and multiplication, neural network morphogenesis, organelle biogenesis, processing of antigens, stress-response pathway, ribosome biogenesis, degeneration of neurons and muscular cells, plant hormone regulation, and viral infection. Ubiquitination is most commonly thought to be associated with the degradation of proteins by the proteasome.

The ubiquitin system is associated with the regulation of the cell cycle. Later, it was discovered to belong to the evolutionarily conserved ubiquitin-conjugating E2 enzymes (Goebl et al., 1988). It has been shown that ubiquitin-mediated proteolysis of cyclin is necessary for cells to leave mitosis (Glotzer et al., 1991). The E3 enzyme plays a crucial part in the separation of chromosomes during mitosis and meiosis (Nasmyth, 2001). Any mistake during the separation of chromosomes during meiosis or mitosis can cause an altered number of chromosomes, which can lead to miscarriage. An additional chromosome 21 in humans also causes Down syndrome, which is due to E3 enzyme degradation by ubiquitin-mediated proteolysis (Honda et al., 1997). The effects
of silencing ubiquitin conjugating enzyme in higher plants are, however, are not well understood.

**The Ubiquitin-Conjugating System**

The ubiquitin-conjugating system is a series of complex enzymatic cascade reactions composed of ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin-ligating enzyme (E3). Ubiquitination is reversible, like the process of phosphorylation and dephosphorylation. Most mammalian genomes encode just two E1 enzymes, about forty E2 enzymes, and more than 400 putative E3 ligases that provide substrate specificity (Randow and Lehner, 2009; Alcaide-Loridan and Jupin, 2012). The identification of the E3 ligase is essential to understand because of its use in drug development for treatment of human diseases. Because of this capability, ubiquitin is a very attractive target, not only for pharmaceutical companies, but also for viral pathogens.

**Plant Viruses and Ubiquitin System**

Different studies showed that plant viruses, especially positive-strand RNA viruses, appear to be interacting with the ubiquitin-proteasome system. The ubiquitin proteasome system has an impact on immune responses. This system is an important part of host defense response against viral infections in plant immunity (Trujilo and Shirasu, 2010). Ubiquitination in plants has been studied most completely in *Arabidopsis thaliana*, in which approximately 5% of the total translated proteins are involved in the ubiquitin-proteasome pathway (UPP). In Arabidopsis, thirty-seven of those genes code E2s, and eight code E2-like proteins (Smalle and Vierstra, 2004). Ubiquitin plays an important role in *Tomato bush stunt virus* (TBSV) infection. TBSV replication protein p33 interacted with host E2 ubiquitin conjugating enzymes, which were
Rad6/ubc2 and cdc34p. Deletion of these proteins interfered with virus replication, showing a direct role for these factors in promoting virus infection (Li et al., 2008).

Cotton leaf curl Multan virus (CLCuMV), which is in the Geminiviridae family and is a member of the genus Begomovirus, encodes a single gene βC1. This gene is a pathogenicity protein and is important for typical symptom expression. It has been shown that the DNA-β-specific symptoms of the host are induced by the interaction between βC1 and the tomato E2 (S1UBC3) of the ubiquitin system (Eini et al., 2009). In order to modify the host UPP, βC1 binds to S1UBC3 on the myristoylation-like motif. The result shows that the E2 is a target of virus. With the help of the target protein, the virus can succeed in escaping host defenses or exploiting host biological pathways (Liang et al., 2013).

All of this is evidence for the ubiquitin systems and shows its importance and vital roles in regulating many different pathways, including protein degradation, protein trafficking, transcription, cell-cycle control, and cell signaling (Mukhopadhyay and Riezman, 2007). There is strong evidence to suggest that viruses take advantage of the host’s ubiquitination pathway (Shackelford et al., 2004; Shackelford et al., 2005).

**Cyclophilins**

Cyclophilins are a group of proteins which have been shown to have peptidyl-prolyl cis-trans isomerase (PPIase) activity. The major PPIase activity involves proteins in protein folding and is mostly classified into three major classes: parvulins, FK506 binding proteins (FKBP), and cyclophilins. A FKBP and CYP group called immunophilins were identified as receptors for immunosuppressive drugs (Harding et al., 1989; Takahashi et al., 1989). Cyclophilins’ key functions in the cell are an assembly of multidomain proteins, muscle differentiation, protein folding, detoxification of reactive
oxygen species (ROS), and immune responses for various diseases—for instance, atherosclerosis, cancer, diabetes, and neurodegenerative disease (Wang and Heitman, 2005; Galat and Bua, 2010; Lee and Kim, 2010).

Cyclophilins are classified under the Enzyme Commission (EC) number 5.1.2.8. Cyclophilins are structurally conserved during evolution, and all have PPIase activity (Wang and Heitman, 2005). Cyclophilins exist in all subcellular section ubiquitous proteins (Galat, 1999). Research showed that they are in present in plants, insects, bacteria, fungi, and mammals (Wang and Heitman, 2005).

**Discovery of Cyclophilins**

In 1984, the first protein was identified that showed PPIase activity (Fischer et al., 1984) and other studies showed the purification of cyclophilin A (CypA), an intercellular protein that binds the immunosuppressant cyclosporin A (Handscharumacher et al., 1984). Later studies showed that PPIase and CypA were the same (Fischer et al., 1989; Takahaski et al., 1989); these studies came after other PPIase proteins (immunophilins) were discovered—for example, the FK-506-binding protein (FKBP). In 1990, the first plant cyclophilins were identified from corn, tomato, and rapeseed (Gasser et al., 1990).

All cyclophilins share a common 109aa (amino acids), a cyclophilin-like domain (CLD), and unique domains to each member of the family (Wang and Heitman, 2005).

**Structure and Function of Cyclophilins**

As previously mentioned, cyclophilins’ PPIase activity by CLD domains are vital for the selection of protein substrates and subcellular compartmentalization (Arevalo-Rodriguez et al., 2004; Galat, 2004; Galat and Bua, 2010). The human genome contains sixteen cyclophilin isoforms that have different cellular and tissue distribution.
Seven major cyclophilins are hCypA, hCypB, hCypC, hCypD, hCypE, hCyp40 and hCypNK.

In recent years, cyclophilins’ identification and characterization studies have progressed drastically. Cyclophilins have key functions in RNA virus replication. Cyclophilins’ inhibitor peptide cyclosporine (CsA) showed the inhibitor factor of HepatitisC Virus (HCV) in cell culture (Nakagawa et al., 2004). It also has a potential effect on the viral RdRp or RNA template that might alter the template activity of RdRp (Watashi et al., 2005). Cyclophilins have been shown to have antiviral activity to influenza A by binding to the matrix protein of the influenza A virus, which interfered with the nuclear localization of matrix protein (Liu et al., 2009). Others found that cyclophilin inhibits the infectivity of human immunodeficiency virus-1 (HIV-1) (Sokolskaja and Luban, 2006; Strebel et al., 2009). Cyclophilin D has become new target for drug testing because of its potential to be a therapeutic agent in many diseases (Waldmeier et al., 2003).

Cyclophilins have been found in cellular parts of most tissues (Galat, 2003; Waldmeier et al., 2003). Those studies revealed that different organisms have a number of genes that contain several cyclophilins. For example, Drosophila has at least nine cyclophilins (Galat, 2003), Caenorhabditis elegans has eleven cyclophilins (Page et al., 1996), and Saccharomyces cerevisiae has eight cyclophilins (Arevalo-Rodriguez et al., 2004). In Arabidopsis, twenty-nine cyclophilins and twenty-three FKBP s have been found to encode putative immunophilins activity from a total of fifty-two genes (Zengyong et al., 2004). Cyclophilins play a very important role in stabilizing the cis-trans transition state and accelerate isomerization, a process in protein folding during
the assembly of multidomain proteins (Gothel et al., 1999). Certainly, some protein-folding processes depend on the catalytic or chaperone-like activities of cyclophilins—for example, human immunodeficiency virus (HIV)-1 formation and the infection progress with the help from CypA (Luban, 1996).

The fruit fly *Drosophila melanogaster* contains a retina-specific cyclophilin, NinaA (an ortholog of mammal CypC), which is necessary for the folding of rhodopsin isoforms. Any mutation of encoding of the gene resulted in improper folding of rhodopsin and caused abnormal expression of the protein (Stamnes et al., 1991). Mammalian retinas also have cyclophilins. The study showed that bovine cyclophilins’ functions, like the NinaA protein, assist proper folding or intracellular transport of opsins (Ferreira et al., 1995).

Cyclophilin expression is involved in responding to stress and possibly functions in stress tolerance in plants. Rotamase Cyclophilin (ROC1, ROC2, and ROC3) expression increases after wounding in the Arabidopsis (Chou and Gasser, 1997). Other examples in the maize and bean cyclophilin gene-expression-level changes respond to heat stress, wounding, high salinity, or low temperature (Marivet et al., 1994). Cyclophilin gene expression was increased by low temperature, abscisic acid, drought, or wounding in the *Solanum commersonii* (Meza-Zepeda et al., 1998). Cyclophilin gene also was upregulated by fungal infection and abiotic stress conditions in *Solanum tuberosum* (Godoy et al., 2000). Cyclophilin expression in the pepper was differently regulated during abiotic stress and pathogen infection (Kong et al., 2001). The study showed that the citrus canker, (*Xanthomonas axonopodis pv. citri*) type III effector protein PthA interacts with citrus cyclophilin (Domingues et al., 2010).
Results

The four citrus genes were identified as CTV-binding proteins, and these genes were targeted for silencing using the CTV-T36 based vector. To date, the identities of the plant genes affecting the aphid transmission of CTV were totally unknown.

Identification of the Plant Proteins with which CTV binds

The affinity of CTV for citrus proteins was previously identified by a Far-Western experiment by my advisor and detailed in Chapter 2. The following proteins were identified and studied:

1. A miraculin-like protein-1 [Citrus jambhiri] named RNAi#1;
2. An iron superoxide dismutase, partial [Citrus maxima] (SOD) named RNAi#2;
3. A ubiquitin-conjugating enzyme variant [Citrus sinensis] (E2) named RNAi#3; and

Building Hybrids to Silence Genes Encoding the Citrus Binding to CTV Particles

Here, I examined the effects of silencing plant genes on aphid transmission through T36 isolates of CTV that are genetically distinct and have very low aphid-transmissibility (around 1%). CTV has been studied as a viral-expression vector (El-Mohtar and Dawson 2014). Many clones of T36 expression vector were engineered and inserted between p23 and 3'-NTR, showing the CTV gene-silencing ability. With this knowledge, I cloned citrus-plant genes which were identified by Far-Western. Therefore, I engineered truncated citrus genes of miraculin-like protein 1(RNAi#1), iron superoxide dismutase (RNAi#2), ubiquitin-conjugating enzyme (RNAi#3), and cyclophilin (RNAi#4) in between p23 and 3'-NTR. I used two different cDNA clones of CTV-plasmid vectors as a backbone; therefore, one of the plasmids had GFP, and another vector plasmid did not contain GFP. Remarkably, I was able to build seven new clones, except for RNAi#3 (E2-ubiquitin conjugation enzyme) without-GFP (Figure 2-5. and Figure 2-4). It was
more challenging to clone a ubiquitin conjugation enzyme that than the other three RNAi. It was affecting the *E. coli* transformation; as a result of that, I was able to obtain five colonies and only one had the correct profile and sequencing.

**Phenotype Result of Gene Silencing**

During the infection in *N. benthamiana*, the virus accumulation period of these constructs was a typical infectious stage of CTV and was photographed and showed in pictures, seen in Figure 4-1 and Figure 4-2.

After the carrying these engineered RNAi#1 (miraculin-like protein 1), RNAi#2 (SOD-iron superoxide dismutase), RNAi#3 (E2-ubiquitin-conjugating enzyme) and RNA#4 (cyclophilin) plasmids to citrus, I examined the set of observable characteristics of CTV and compared them to the T36 isolate of CTV-infected citrus plants. From these four silenced-genes, SOD and E2 showed phenotype, and SOD showed very light symptoms, which was light leaf chlorosis, the most common and obvious symptom of iron deficiency in plants. This mild symptom disappears as the leaves and tree age. Chlorotic-bleaching phenotypes were also the result of the vector silencing of ubiquitin conjugation enzymes. The infected leaf abaxial and adaxial surfaces showed the symptoms of bleaching and some location of chlorotic spots. In addition to this, the new flush exhibited white-bleached symptoms, necrosis, and leaf drop with light-green bark plus dark-browning tissue. The results of these symptoms are presented in Figure 4-3. The silencing of miraculin-like protein-1 and cyclophilin did not show any phenotype differences than CTV-infected *C. macrophylla*. 


**CTV Replication and Stability**

To determine the stability of these silencing hybrids, the titer of CTV infectious clones was determined by real-time RT-qPCR and ELISA in flush tissue of *C. macrophylla* seedlings which were used as aphid-transmission sources. The titers were compared to the unmodified T36 infectious clone (control).

The virus titer after silencing miraculin-like protein 1, iron superoxide dismutase, ubiquitin-conjugating enzyme and cyclophilin infectious clones showed slightly higher levels than T36 isolates of CTV infectious clones, as determined by a real time RT-qPCR quantification of viral RNA by RT-qPCR. Table 4-1 showed that there was no significant differences (Tukey’s HSD; P>0.05). ELISA was also performed with dilution serial and showed similar results (Figure 4-4).

**Aphid Transmission of CTV**

To understand the effect of silencing these four gene constructs on aphid transmission, four new *C. macrophylla* seedling plants were graft-propagated for aphid transmission to have enough donor plants. All hybrids were inoculated into *C. macrophylla* seedlings, and successful inoculation was confirmed by ELISA.

The aphid transmissions were completed using a total number of 101 plants for RNAi#1 clone with five replications, 90 plants for RNAi#2 clone with five replications, 46 plants for RNAi#3 clone with four replications, and 54 plants for RNAi#4 with four replications. The results were that these silencing CTV clones transmitted at the rate of 0% to 22.22% for the RNAi#1 (miraculin-like protein1), and 4% to 50% for the RNAi#2 (iron superoxide dismutase), 0% for RNAi#3 (ubiquitin-conjugation enzyme), and 0% to 25% for the RNAi#4 (cyclophilin) (Table 4-2). The transmission rate of the control, CTV T36 cDNA clone, was 1.5%.
Remarkably, the silencing of specific plant genes changed the aphid transmission rate of CTV. Therefore, the aphid transmission rate was 5.94% for RNAi#1 (miraculin-like protein-1), 24.44% for RNAi#2 (iron superoxide dismutase), 0% for RNAi#3 (ubiquitin-conjugation enzyme), and 7.41% for RNAi#4 (cyclophilin).

**Discussion**

Virus-induced gene silencing (VIGS) is a powerful and promising tool that offers a fast process for identifying gene function in plants. VIGS technique is useful for suppressing endogenous gene expression by infecting plants with a recombinant virus vector (VIGS vector) carrying host-derived sequence (Senthil-Kumar and Mysore, 2011). As mentioned in chapter 1, there is a possible use of CTV-based vector as an experimental tool. Dr. Dawson’s laboratory showed that CTV could be an efficient expression vector capable of expressing multiple foreign genes engineered at different positions in its genome, either as extra genes or as a substitution of some nonessential genes using homologous and heterologous subgenomic RNA (sgRNA) controller elements (Dawson and Folimonova, 2013; El-Mohtar and Dawson, 2014). Here, I described and used an approach based on discoveries concerning plant viruses and gene silencing. Virus-based technology has been a useful complement to existing functional genomics tools. The T36-CTV vector was used to develop a construct for the silencing of the citrus plant genes miraculin-like protein 1, iron superoxide dismutase, ubiquitin-conjugating enzyme, and cyclophilin. These four citrus proteins were identified by a Far-Western experiment through CTV binding to plants’ genes.
Building Hybrids to Silence Genes Encoding the Citrus Binding Proteins to CTV Particles

Citrus-gene silencing has been made possible by engineering sequences of interest at the 3'-end of the CTV. With the RNAi constructs, the gene-silencing capabilities of CTV vectors has been demonstrated in *C. macrophylla* plants. These hybrids were also stable in the plants as shown using GFP (Figure 4-2).

**Phenotype of Silencing**

In the citrus plant, the most visible symptom of iron deficiency is a characteristic chlorotic leaf pattern. It appears on young leaves first and then on expanding leaves. The whole leaf looks light green or yellow, except that the fine network of midrib and veins stays dark green (Timmer et al. 2000: Fu et al. 2017). In this experiment, greenhouse plants were under a routine micronutrient fertilization schedule; most likely because of that, this mild iron deficiency symptom disappeared. RNA#3-infected *C. macrophylla* plants showed a photo-bleaching phenotype in the newly emerging leaves, stems, and thorns and some chlorotic spots on the older leaves (Figure 4-3).

**Aphid Transmission of CTV**

This was the first report on the effects of silencing plant genes on aphid transmission of CTV. As a result of gene silencing, the aphid transmission rate of CTV increased compared to the natural infection rate. Silencing these proteins affected transmission differently; their effects originated from different modes. They should be approached separately.

After silencing miraculin-like protein 1, aphid transmission of CTV was about 6%. The low range of the aphid-transmission rate at the beginning of experiment was most likely due to the establishment of CTV, and the silencing of plant genes takes
processing time in the citrus. Miraculin is known as a sour-test changer molecule, which attaches to the taste-receptor cells found on taste buds lining the mouth, tongue, throat, and esophagus. Miraculin binds directly to the hT1R2-hT1R3 sweet-sensing taste receptors as an antagonist at neutral pH and set off its taste-modifying activity (Koizumi et al., 2011). Webster et al., (2018) report the identification of two cuticular proteins within aphid mouthparts, which are Stylin-01 and Stylin-02, and for the role of Stylin-01 as the receptor of CaMV. This raises future research questions to answer; there is a possibility that plant genes’ attachment to the aphid receptors affect factors in the transmission of a noncirculative virus. It is unknown whether miraculin attaches to any receptor in aphids, but it is probable that miraculin may be attached to the aphid’s stylet, causing the CTV virion not to attach.

It is worth noting that silencing of superoxide dismutase (SOD) in citrus plants increased the transmission rate of the T36 isolate by aphid from 1.5% (my results), or 0.6% according to Harper et al. (2016), to 24.44%. The superoxide dismutase is well known to constitute the first line of defense against reactive oxygen species (ROS) within a cell (Alscher et al., 2002). The Tomato bushy stunt virus (TBSV) infection resulted in increased superoxide dismutase in roots and leaves of N. benthamiana. Additionally, the virus infection significantly affects enzymes responsible for the balance of ROS accumulation in plant tissue in response to pathogen attacks (Yergaliyev et al., 2016). Increased oxidative stress and changes in SOD gene activities were observed in CTV-infected sweet orange (Dória et al. 2015). Another study suggested that the iron superoxide dismutase protected plants from winter freezing (McKersie et al. 2000). Similarly, ROS plays a critical role in complex plant-insect interactions (Morgan et al.,
2002; Kuśnierczyk et al., 2008). Sytykiewicz et al., (2014) demonstrated that aphid species altered the expression of several similar genes in a density- and time-dependent way in maize seedling; most of the genes examined also were more noticeably upregulated in the relatively resistant-to-aphid maize plants than in the aphid-susceptible maize plants.

Ubiquitin-conjugating enzyme sequencing of *Citrus sinensis* cultivar Pera (627nt) was cloned into T36 infectious clone of CTV. This was the CTV construct that displayed more notable phenotypes; however, it resulted in nonaphid transmissions. To determine whether differences in infection efficacy of the ubiquitin silencing hybrid were caused by differences in viral loads in plants, I tested the CTV titer in the plants using real-time RT-qPCR, as previously described (Harper et al., 2014). Interestingly, relative quantification showed that this construct accumulated slightly more than T36 isolates of CTV; similar results also showed that GFP expression in the leaf was increased; however, results showed that there was statistically no significant differences in CTV replication.

It is known that viruses manipulate the host-cell machinery for their own advantage. Viral replication is dependent on the activity of several host factors. Ubiquitylation has an important role in regulating many different pathways, such as protein degradation, protein trafficking, transcription, cell-cycle control, and cell signaling (Pickart 2004; Mukhopadhyay and Riezman, 2007; Randow and Lehner, 2009). Ubiquitylation is directly antiviral or helpful to the viruses; depending on the cellular context, ubiquitin attachment may either promote or inhibit the viral life cycle. Therefore, viruses have developed means to enhance or inhibit ubiquitination of specific substrates, depending on their needs. It is possible that plant-ubiquitin system inhibits
the CTV advancement in the plant, and this could be the aim of the future studies: to understand the CTV-ubiquitin relationship.

Cyclophilin-silencing-engineered CTV plasmid was affected by increased aphid transmission increase rate. The expression of cyclophilin changed during pathogen infection in pepper, heat stress, wounding, and high salinity in bean and maize (Marivet et al., 1994), and fungal infection and abiotic stress in potatoes (Kong et al., 2001). Cyclophilin expression is involved in the response to stress and possibly functions in stress tolerance in plants.

There is a growing number of publications about viruses, especially those transmitted by aphids and whiteflies, indicating that possible viral infection is able to indirectly influence vector behavior for the efficiency of insect-mediated virus transmission (Colvin et al. 2006; Zarate et al. 2007; Zhang et al. 2013; Li et al. 2017). In plant hosts, virus-induced changes in phytohormone signaling were observed. For instance, Cassava mosaic disease (CMD) infected plants have significantly higher *Bemisia tabaci* (*B. tabaci*-whitefly) population-growth rates than the uninfected plants (Colvin et al., 2006). This shows how the virus improves host quality to facilitate increased infestation and reduces the resistance of plants to whiteflies by suppressing and stimulating certain plant-defense pathways. Viruses have become involved in many ways to inhibit jasmone acid’s (JA’s) responsive gene expression with change regulating salicylic acid (SA)-mediated resistance. Whitefly feeding stimulates SA biosynthesis and triggers the defense signaling that suppresses many JA stimulated anti-insect responses (Zarate et al. 2007; Zhang et al. 2013). In the case of tomato yellow leaf curl China virus (TYCCNV), transmitted by *B. tabaci* that showed prior
feeding by whiteflies on tobacco and tomato, the plants were less susceptible to subsequent inoculation (Li et al., 2017). Plant-secreted volatiles play an important role in attracting aphids to establish for prolonged periods on barley yellow dwarf viruses (BYDV) and potato leafroll virus (PLRV) (Bosque-Prez and Eigenbrode. 2011; Eigenbrode et al. 2002).

In this study, aphids fed on CTV-infected source plants with a time limitation of twenty-four h. There was no report showing that plant genes have direct influences on the efficiency of insect-mediated virus transmission. These discoveries come with new questions for future research. Is the immunity-compromised citrus plant the source of spreading the non- or low transmissible isolate of CTV? Is it possible that the transmission of citrus greening, also known as Huanglongbing (HLB), spread by Asian citrus psyllids feeding from immune-deficient plants at a higher percentage rate than from healthy plants?

In the future, the CTV vector can be used as a powerful tool to understand plants’ immunity through new approaches for silencing genes involved in metabolism, growth, and virulence factors. Another interesting area of research in the future is to understand host resistance strategies for limiting the nutrient availability to pathogens. In vivo gene-silencing is a new approach to study the role of citrus genes as components of the CTV-aphid transmission complex. Moreover, beyond the case of CTV transmission by aphid vectors, I illustrated the complexity of virus-vector, virus-plants host interactions.

Therefore, these findings provide new insights into influencing factors on the plant-virus transmission and discusses potential future research on the tripartite interaction between viruses, host plants, and insect vectors of virus. The tripartite
interactions have further impact on management of the virus diseases, the insect vector, and the host plant.
Figure 4-1. cDNA clone of T36 and CTV gene-silencing construct expressions in *N. benthamiana*. The plants on the left side of the pictures A) Base clone used for silencing hybrids in cloning 35s105; B) Miraculin-silenced hybrid; C) FeSOD-silenced hybrid; D) Ubiquitin-conjugation enzyme-silenced hybrid; E) Cyclophilin-silenced hybrid expression. Photos February 17, 2019. Courtesy of Cecile Robertson.
Figure 4-2. cDNA clone of T36 and CTV gene-silencing construct expressions in Citrus macrophylla. The plants on the left side of the pictures A) Base clone used for silencing hybrids in cloning 35s105; B) Miraculin-silenced hybrid; C) FeSOD-silenced hybrid; D) Ubiquitin-conjugation enzyme-silenced hybrid; E) Cyclophilin-silenced hybrid expression. Photos January 22, 2019.Courtesy of Turksen Shilts.
Figure 4-3. T36 strain of CTV and Ubiquitin-CTV gene-silencing construct expressions in *C. macrophylla*. The plants on the left side of the pictures A, B) T36 strain and chlorotic-bleaching phenotypes are vector silencing of Ubiquitin vector. The infected leaf abaxial (back) surface C) and adaxial (upper) surface D) showing the symptoms of bleaching. Arrows indicate chlorotic spots. The new flush E) showing white-bleached symptoms and leaf drop. The arrows on the picture show white-to-brown bark tissue that was dying. Photos July 24, 2015. Courtesy of Cecile Roberson
Tukey’s Honestly Significant Difference test showed no significant differences between treatments ($P>0.05$).

<table>
<thead>
<tr>
<th>ELISA</th>
<th>Ct Mean/SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Miraculin</td>
<td>3.32±0.07</td>
</tr>
<tr>
<td>Iron superoxide</td>
<td>3.25±0.16</td>
</tr>
<tr>
<td>Cyclophilin</td>
<td>3.22±1.71</td>
</tr>
<tr>
<td>Ubiquitin</td>
<td>3.47±0.10</td>
</tr>
<tr>
<td>T36</td>
<td>3.41±0.05</td>
</tr>
</tbody>
</table>
Table 4-2. Results from CTV infectious clone hybrids that silenced plant genes were used for aphid transmission. Table shows number of replicates, number of plants tested for ELISA, number of plants that became positive, and percentage of transmission.

<table>
<thead>
<tr>
<th>Constructs Name</th>
<th>Number of replicates</th>
<th>Total Plant Tested ELISA</th>
<th>Number of plants positive</th>
<th>Percentage of transmission</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNAi#1 (Miraculin-like protein 1)</td>
<td>1</td>
<td>24</td>
<td>1</td>
<td>5.94 %</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>24</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>10</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>25</td>
<td>1</td>
<td>6 positives out of 101</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>18</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>RNAi#2 (FeSOD-Iron superoxide dismutase)</td>
<td>1</td>
<td>25</td>
<td>1</td>
<td>24.44 %</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>23</td>
<td>4</td>
<td>22 positives out of 90</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>17</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>25</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>RNAi#3 (Ubiquitin-conjugation enzyme)</td>
<td>1</td>
<td>25</td>
<td>0</td>
<td>0 %</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>7</td>
<td>0</td>
<td>0 positives out of 47</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>8</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>6</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>RNAi#4 (Cyclophilin)</td>
<td>1</td>
<td>24</td>
<td>0</td>
<td>7.41 %</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>6</td>
<td>1</td>
<td>4 positives out of 54</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>8</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>16</td>
<td>3</td>
<td></td>
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Figure 4-4. Serial dilutions of ELISA of CTV- RNAi hybrids. B) ELISA read after 60min and A) showing as a graph view. RNAi#1-Miraculin-like protein 1; FeSOD-Iron superoxide dismutase; RNAi#3-Ubiquitin-conjugation enzyme; RNAi#4-Cyclophilin
Viruses are obligate parasites, and a very important part of the survival of plant viruses is to move to new hosts. Viral transmission is the process through which viruses spread to new hosts. Plant viruses are transmitted through budwood, seeds, or tubers or by arthropods, nematodes, or fungi vectors. Most plant viruses are transmitted by arthropods. Despite modern technological advances, identifying the molecular mechanisms of vector transmissions of plant viruses has not been straightforward. It is known that interactions between viruses and vectors are very specific and are unique to each virus.

*Citrus tristeza virus* (CTV) is a major pathogen that affects citrus and has been responsible for the loss of over 100 million trees. This virus is transmitted in a semipersistent manner only by the aphid species or by grafting of infected tissue (Bar-Joseph et al., 1989). *Toxoptera citricida* is the most efficient vector species of most CTV strains, but the molecular mechanisms of transmission remain unclear.

Here, I examined the 5`end genes from different isolates of CTV for aphid vector transmission. To make matters more complex, in addition to these different strains, the rates of aphid transmission rate vary for different isolates of the strains. However, no work had been carried out to determine if replication-associated proteins are involved in aphid transmission. I cloned the different 5`end genes from T68 and T30 isolates of CTV into our T36-based infectious clone, which is very poorly transmitted by the aphid vector. Interestingly, interchanging the entire 5`half of the genome produced a substantial increase in transmission by the aphid vector, from 1.5% transmission efficacy to 23.20% with the T68 substitution. In a further step, I cloned a single gene
from the T68 isolate to the T36 infectious clone, which generated surprising results. Swapping the p33 gene from the T68 to the T36 strain increased the aphid transmission rate to 17.78%. This was the first evidence to suggest that the p33 gene is one of the key components of CTV aphid transmission.

For these studies I used viral-induced gene silencing (VIGS), a technique that utilizes recombinant viruses to specifically reduce endogenous gene activity through plant innate silencing mechanisms (Voinnet 2001). The technique has been used widely to analyze the gene function of plants and has been adapted for high-throughput functional genomics. Although gene silencing is still in an early stage of application, VIGS offers a new perspective for understanding the transmission process and the intimate relationship between virus, host, and vector. It can be used to design novel control strategies against plant pathogens and plant viruses, such as the genetic manipulation of vectors and the expression of proteins in plants to neutralize the transmission process.

It has been shown that the manipulation of the CTV infectious clone can be developed to create a stable vector to express foreign sequences or silence endogenous plant genes. A vector based on the T36 strain of CTV has been developed for transient expression of foreign genes in citrus trees using a green fluorescent protein (GFP) as a reporter.

In order to understand the molecular mechanisms of the transmission of CTV by aphids, I screened putative CTV-binding proteins/molecules from citrus plants using the gene-silencing ability of CTV in citrus. The proteins studied were a miraculin-like protein-1, an iron superoxide dismutase, a ubiquitin-conjugating enzyme variant, and a
cyclophilin. These four genes were targeted for silencing using the CTV-T36-based vector. These CTV gene-silencing construct phenotypes were observed in infected *N. benthamiana* and *C. macrophylla* plants. Common CTV infection was observed in the *N. benthamiana* plants, and no unusual phenotype was observed in any of the four constructs.

In the citrus plant, some unusual phenotype observed in the iron superoxide dismutase silenced hybrid. The most noticeable symptom was chlorotic leaf pattern that is a characteristic in iron deficiency plants. On the ubiquitin-conjugating enzyme silencing hybrid, a photo-bleaching phenotype was found in the newly emerging leaves, stems, and thorns of the infected *C. macrophylla* plants, and some chlorotic spots were found on the older leaves. The silencing of miraculin-like protein-1 and cyclophilin did not show any phenotype differences than CTV-infected *C. macrophylla*. The silencing action of miraculin-like protein-1 and cyclophilin did not produce any phenotype differences in the CTV-infected *C. macrophylla*.

The question remains why CTV has to interact with plant genes that it does not seem to need. It is possible that these genes may be involved in insect vector transmission of CTV. With this possibility in mind, I conducted an aphid transmission experiment using these CTV-silencing hybrids.

Three of the plant-gene-silencing CTV constructs, the miraculin-like protein-1, the iron superoxide dismutase, and the cyclophilin, increased aphid transmission by 5.94%, 24.44%, and 7.41%, respectively. The ubiquitin-conjugating enzyme variant, resulted in 0% aphid transmission. This is the first study to show that citrus plant genes have
influence on aphid transmission of CTV. There was no report showing that plant genes have direct influences on the efficiency of insect-mediated virus transmission.

Several publications on viruses indicate that viral infection may be able to indirectly influence vector behavior to increase the efficiency of insect-mediated virus transmission. Virus-induced changes in phytohormone signaling have been observed in plant hosts. Viruses also indirectly impact plant-secreted volatiles, which play an important role in attracting aphids to establish themselves for prolonged periods on some viruses. A clear understanding of virus-vector interactions during transmission requires an understanding of virus interactions with the host plant.

I have illustrated the potential complexity of virus-vector, virus-host plants relationships. Understanding the biological and molecular interactions between the CTV virus and its aphid vector is important for future disease control. The CTV vector can be used as a powerful tool for studying plant immunity and for developing new approaches to silencing genes involved in metabolism, growth, and virulence factors.
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BIOGRAPHICAL SKETCH

Turksen Shilts was born in Eski Cuma, Bulgaria. She received her Bachelor of Science in agricultural study from Bursa Uludağ University, Faculty of Agriculture. Upon graduation, she worked at the Beta Seed Corporation. While she was continuing her education. She had the opportunity to study at the Technical University of Munich (TUM) as a summer internship with the DAAD (Deutscher Akademischer Austauschdienst) scholarship. Her graduate research focused on physiology of the dormancy of kiwi fruit. She received Master of Science degree from Bursa Uludağ University. She worked in Gardenia AS flower production and landscaping after she graduated before relocated to the USA.

After moving to Florida, she joined the work at University of Florida, Citrus Research Center. During this period, she participated in research projects and experiments related to exotic citrus pathogens, learning various molecular biology techniques, molecular cloning and sequencing of pathogens, genomes as well as working with greenhouse plants on their inoculation with pathogens and analysis using Real time-PCR and other diagnostic methods along with other team members in the lab. In her current position she focused on Citrus tristeza virus. She graduated from University of Florida with PhD from Plant Pathology Department in 2019.