

RNA INTERFERENCE–BASED STRATEGY FOR ASIAN CITRUS PSYLLID
(*DIAPHORINA CITRI* KUWAYAMA) CONTROL: A METHOD TO REDUCE THE
SPREAD OF CITRUS GREENING DISEASE (HUANGLONGBING).

By

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To my Mum and Dad

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LIST OF ABBREVIATIONS

ACP	Asian citrus psyllid
AGO	Argonaute
BYV	Beet yellow virus
CE	Controller element
<i>C. elegans</i>	<i>Caenorhabditis elegans</i>
<i>C.L.asiaticus</i>	<i>Candidatus Liberibacter asiaticus</i>
C. Mac	Citrus Macrophylla
CP	Coat protein
CTV	Citrus tristeza virus
dsRBM	Double stranded RNA binding motif
dsRNA	Double stranded RNA
<i>E. coli</i>	<i>Escherichia coli</i>
EDTA	Ethylenediaminetetraacetic acid
<i>E. postvittana</i>	<i>Epiphyas postvittana</i>
EtBr	Ethidium bromide
ETOH	Ethanol
GFP	Green Fluorescent Protein
HEL	Helicase
HLB	Huanglongbing/ Citrus greening disease
LB	Luria broth
mRNA	Messenger RNA
MT	Methyltransferase
NaCl	Sodium Chloride
NaOAc	Sodium Acetate

ORF	Open reading frame
PCR	Polymerase chain reaction
PRO	Protease
PTC-100	Peltier Thermal Cyclers - 100
qRT-PCR	Quantitative reverse transcriptase polymerase chain reaction
RdRp	RNA dependent RNA polymerase
RISC	RNA – induced silencing complex
RNAi	RNA interference
sgRNA	Sub genomic RNA
SID	Systemic-interference-defective
siRNA	Small interfering RNA
S.O.C	Super Optimal Broth with Catabolite repression
ssRNA	Single stranded RNA
TAE	Tris-acetate-EDTA
TG	Target gene
UV	Ultra-violet

Abstract of Thesis Presented to the Graduate School
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The Asian citrus psyllid (ACP), *Diaphorina citri* Kuwayama, is a worldwide citrus pest. ACP transmits a bacterium that infects citrus phloem, causing citrus greening disease (Huanglongbing, HLB) infecting citrus phloem. Disease symptoms include yellow leaves and veins, premature fruit drop, plant dieback, and death, all resulting in major economic burdens. Current efforts focus on limiting disease spread using RNA interference (RNAi) as an approach to induce ACP mortality. RNAi is a cellular defense mechanism in eukaryotes targeting invading viruses producing double-stranded RNA (dsRNA). The dsRNA that enters the cell is cleaved into small interfering RNA (siRNA), 21-23 base pairs long, by an endonuclease called DICER. One siRNA strand binds to a protein complex called RNA-induced silencing complex (RISC). This molecule scans the cellular cytoplasm for mRNA sequences that are complementary to the incorporated siRNA strand, and when encountered, the mRNA is cleaved, becoming non-functional, inhibiting protein biosynthesis. Control of an insect is accomplished by feeding the insect dsRNA molecules with a sequence complementary to that of the mRNA of an essential insect gene. A plant expression vector constructed from Citrus tristeza virus

(CTV) was used for ACP to ingestion of specific dsRNA. CTV is a phloem-limited single stranded RNA virus that produces large numbers of dsRNA replicative forms in the phloem sieve elements and companion cells of citrus plants. An infectious non-pathogenic CTV strain was engineered to produce dsRNA complementary to an essential ACP gene, target gene sequence 1 (TG1), and used to infect *Citrus macrophylla*. Following infection, cuttings were analyzed showing that the phloem contained many copies of ssRNA and dsRNA TG1 fragments. The number of TG1 sequences varied between individual leaves of infected plants. The younger flush stages, preferred by ACP, had the highest TG1 sequences. Feeding CTV/TG1 infected ACP plants caused 70% mortality suggesting a correlation between dsRNA copies and ACP mortality. Oviposition activity however was not affected. This research therefore, presents evidence that ACP mortality can be induced through oral uptake of dsRNA targeting specific ACP mRNAs and thus blocking the spread of citrus greening disease.

CHAPTER 1 INTRODUCTION

Citrus greening disease, or Huanglongbing (HLB) is considered the most serious disease of citrus worldwide, known to occur in 40 Asian, African, Oceanian, and South and North American countries (Bove, 2006). HLB leads to unmarketable fruit and eventual death of the tree (Childers and Rodgers, 2005), and if infected trees are not removed it can affect entire groves. In 2005, HLB was identified in the state of Florida and has since become widespread throughout the state's commercial production areas (Hodges and Spreen, 2012). The state of Florida is the largest citrus producer in the United States (third worldwide), with the industry representing an important section of the agricultural economy valued at \$8.91 billion and generating at least 75,800 jobs (Hodges et al., 2000).

HLB is vectored by the Asian citrus psyllid (ACP), *Diaphorina citri* Kuwayama, a sap-sucking insect which feeds exclusively on citrus and closely related species. ACP was first detected in Florida in 1998 (Halbert and Manjunath, 2004). With no cure for the disease, current control strategies are based upon vector control to limit the spread.

My research focuses on using an emerging molecular technique termed RNA interference (RNAi) that is a natural cellular defense process that occurs in all eukaryotic organisms which targets foreign, invading double-stranded RNA (dsRNA) molecules, such as viruses. This defense is initiated when foreign double-stranded molecules enter the cell and are recognized and cleaved into small interfering RNA (siRNA) of 21-23 base pairs by an endonuclease called DICER. One of these strands of the siRNA is then bound to another protein, an RNA-induced silencing complex (RISC), where it is used as a template to seek out complementary sequences within the mRNA

population in a sequence specific manner. When the complex binds to a complementary sequence in the mRNA pool, the mRNA is cleaved, making it non-functional or 'silenced' meaning that no cognate protein can be produced. This strategy shows promise as a mechanism for ACP control because studies have shown that feeding ACP dsRNA sequences matching the sequence of its own essential genes causes ACP mortality.

A commercially deployable strategy would be to engineer citrus trees that can produce these dsRNA molecules in the phloem so they are ingested by the psyllid. This was achieved in my research using a citrus viral expression vector, citrus tristeza virus (CTV). CTV is a phloem-limited ssRNA virus that produces dsRNA replicative intermediate forms of the genome and a large number of truncated genomic dsRNA molecules in the phloem sieve elements and companion cells of citrus plants (Karasev et al., 1997). The CTV clone used as a delivery vector is infectious but non-pathogenic to the plant, and engineered to contain ACP's own essential genes, thus causing a down regulation of these genes when feeding on infected citrus. In this thesis the name of the target gene will be referred to as Target Gene 1 (TG1), because sequence of the gene is currently unpublished the information about the gene is proprietary and cannot be disclosed in format.

My overall research goal was to investigate a sustainable method to prevent the spread of HLB by targeting the ACP vector for control, avoiding the use of harmful pesticides, to prevent further destruction to the citrus industry in Florida and other states. My specific objectives were to:

1. Characterize and analyze TG1 RNA vectored to citrus by CTV
2. Quantify the effect of the TG1 dsRNA on ACP mortality, development, and oviposition.

CHAPTER 2 LITERATURE REVIEW

Citrus Greening Disease

Citrus greening disease, or Huanglongbing (HLB), is one of the most serious diseases of citrus worldwide. It was first reported in southern China in 1919 and is now known to occur in 40 Asian, African, Oceanian, and South and North American countries, including the U.S. where it was first discovered in Florida in 2005 (Bove, 2006), but has recently been reported in Texas and California. Prior to 2004, it was responsible for the loss of over 60 million trees worldwide (Graca and Korsten, 2004), however, this has likely increased from March 2004 and August 2005 when symptoms of the disease were recognized in Brazil and Florida the two largest citrus growing regions in the world (Bove, 2006). Huanglongbing, loosely translated as “yellow shoot” disease in English publications, was named because of characteristic yellow shoots (Figure 2-1) caused by the disease (Halbert and Manjunath, 2004),

In addition, infected trees show a blotchy yellow-green mottling of the leaves which is seen during early infection (Bove, 2006). Symptoms also include chlorotic leaves with veins retaining dark coloration (Childers and Rogers, 2005), stunted trees growth and reduced canopy density due to leaf drop, reduced fruit yield, small and deformed unripe fruits with the coloration starting at the peduncular end, resulting in progressively unproductive trees (Halbert and Manjunath, 2004; Bove, 2006). Off-season bloom with fruit drop and twig die-back are also symptomatic. The fruits produced (Figure 2-2) can be small, lopsided, and hard with a bitter taste (Halbert and Manjunath, 2004). Symptoms can also resemble nutritional stress such as zinc

deficiency, which is indistinguishable from HLB symptoms, making symptoms difficult to recognize with certainty (Halbert and Manjunath, 2004; Bove, 2006).

Severe symptoms are usually found between 1 and 5 years after the appearance of the onset from first symptoms. As the disease severity increases, the yield is reduced by early fruit drop from infected branches. Yield reduction can reach 30 to 100%, depending on the proportion of canopy affected, and makes the orchard economically unfeasible in 7-10 years after planting (Gottwald et al., 2007).

The bacteria associated with and presumed to cause HLB belong to the genus *Candidatus Liberibacter*. Three species are known to be transmitted by ACP. *Candidatus Liberibacter asiaticus* was first identified in Asian countries and is now found in Brazil and Florida (Graca, 2008). This microbe is a phloem and sieve-tube restricted gram-negative bacterium. *Candidatus Liberibacter asiaticus* possess very thin walls, which allow them to penetrate through narrow sieve pores. *Candidatus L. asiaticus* is heat tolerant and able to survive at temperatures above 30°C for extended periods (Graca, 2008), but is sensitive to temperatures above 43°C.

The bacterium is naturally transmitted from tree to tree by the ACP which acquires the pathogen while feeding on the phloem of infected citrus. Transmission is circulative, it is taken up and it is suspected that receptors on the ACP gut interact and bind with the bacterium, transporting it from the gut to the hemolymph (Stelinski et al, 2010). It is transported then to the salivary glands where it can be transmitted to a new plant when ACP feeds on the phloem. The bacterium can replicate inside the alimentary and salivary glands in a persistent manner (Ammar et al., 2011)

Asian Citrus Psyllid

The Asian citrus psyllid (ACP) is a major pest of the citrus industry, with preeminent economic damage due to its ability to transmit *Candidatus L. asiaticus*, thus spreading citrus greening disease. In areas where there is no citrus greening disease, ACP is considered a minor pest (Halbert and Manjunath, 2004). The insect is ubiquitous and present in regions where citrus greening is endemic (Bonani et al. 2009). It is widely accepted that the species of bacteria associated with citrus greening multiplies in the ACP vector, however, this has not been definitively demonstrated with molecular evidence.

The Asian citrus psyllid is a phloem-feeder endemic to South-east Asia and thought to have originated here (Chen, 1990). It was first detected in the USA in Palm Beach County, Florida in 1998 (Halbert and Manjunath, 2004) and now occurs throughout Florida. It was reported in Texas in 2001 (French et al., 2001) and in San Diego, California in September 2008. Worldwide it is found in tropical and sub tropical Asia, Afghanistan, Saudi Arabia, Reunion, Mauritius, parts of South and Central America, Mexico, and the West Indies. The ACP needs young, actively growing flush on which to lay eggs and to support nymphal development. In southern Florida, population peaks of ACP occur in May, August, and October through December, which coincides with growth of *Murraya paniculata*. ACP numbers go down after heavy rain and in dry humid seasons; ACP will peak at its highest level after the first growth flushes of the new wet season following dry weather (Bove, 2006).

In terms of visual cues, ACP is innately attracted to bright yellow and green (Sanchez 2008; Wenninger et al., 2009), colors that may be indicative of flushing shoots. ACP is also attracted to the volatiles emitted by the flush of its plant host (Patt

and Setamou, 2010). ACP adults are 3-4 mm long. Most feeding activity occurs on new flush but they can also feed on mature citrus, especially during winter months (Childers and Rogers, 2005). Adults have been found to have significantly higher numbers on sweet orange than on grapefruit (Setamou et al., 2008). Adults live for 1-2 months, with their life span dependent on temperature and host plant (Tsai and Lui, 2000).

Characteristically, ACP adults form a 45° angle (Figure 2-3) with respect to the plane of the plant tissue on which they feed (Bove, 2006). Females oviposit on very young developing flush, on the tips of growing shoots, and on and between unfurling leaves (Bove, 2006). Eggs are about 0.3 mm long, initially pale yellow at deposition but becoming more orange as they mature. The number of eggs produced is dependent upon the host plant, with studies showing more laid on grapefruit than on rough lemon, which could be attributed to the higher amount of flush points (Liu and Tsai, 2000). Average egg incubation periods on Orange Jessamine, grapefruit, rough lemon, and sour orange vary little (Tsai and Lui, 2000).

ACP has five instars (Figure 2-4) that range from 0.25 to 1.7 mm long; they can be identified by their yellow-orange coloration with noticeable red eyes and large lateral wing pads in the fifth instar (Liu and Tsai, 2000). Nymphs feed on phloem in developing flush and while feeding they secrete copious amounts of white, curled waxy honeydew (Ammar et al., 2011). The optimum range of temperatures for ACP population growth is 25 - 28°C (Liu and Tsai, 2000). The nymphs are known to acquire the bacterium associated with citrus greening disease more efficiently than the adults (Qureshi et al., 2009, Stelinski et al., 2010).

Because there is no chemical treatment that will kill the bacterium associated with HLB, focus on controlling HLB has been to target the vector for control. Current control relies upon broad-spectrum pesticides such as the foliar insecticides: Actara, Danitol, Lorsban and Provado, as well as the systemic insecticides: Admire and Temik (Childers and Rogers, 2005). With the growing concern of the broad-spectrum insecticides' impact on the environment and human health, and with the problem of natural enemies being unpredictable and too generalist, alternative control strategies are being sought (Srinivasan, 2010, Michaud, 2002).

RNA Interference

RNA interference (RNAi) is a natural evolutionary-conserved cellular mechanism that directs protection against nucleic acid invaders such as viruses, repetitive sequences, and transposable elements that occur in plants, animals, protozoans and fungi (Aronstein et. al 2011, Fulci et al, 2007). It is a post-transcriptional gene silencing strategy carried out as a rapid cellular process where double-stranded RNA (dsRNA) directs sequence-specific degradation of cognate messenger RNA (mRNA). Although the general concept of gene silencing has been known and studied for many years, Fire et al. (1998) pioneered the use of RNAi as a method to inhibit gene expression in nematodes. In this case, an RNAi response was provoked by injection of the dsRNA into the gonads of *Caenorhabditis elegans* (Maupas 1900) or by introduction of the dsRNA through feeding either of dsRNA itself or bacteria engineered to express it. Exposure to these dsRNA resulted in a loss of the corresponding mRNA and effectively silenced the targets at the post-transcriptional level (Fire et al., 1998). Initial observations were consistent with dsRNA- induced silencing operating at the posttranscriptional level. These findings provided evidence for a more ubiquitous gene

silencing strategy that had been observed in plants for quite some time (Hannon, 2002). These studies have led to the assumption that RNAi induces the degradation of mRNAs that share sequence homology to dsRNA molecules detected within a cell (Hannon, 2002).

The RNAi pathway is first initiated when a double-stranded RNA molecule (typically from virus origin) enters the cellular cytoplasm. The specific method of uptake into the cell and transport throughout the insect is currently unknown and has been the focus of ongoing research (Aronstein et al., 2011). Data suggest that there must be at least two systems involved in dsRNA uptake in insects/invertebrates (Huvenne and Smagghe, 2010). One method is based on the transmembrane *sid-1* (a systemic-interference-defective) channel protein. A single *sid-1* gene has been identified in most sequenced insect genomes. According to this theory, *sid-1* is not needed for dsRNA to enter the intestinal epithelial cells. This is thought to occur by endocytosis of the dsRNA aided by *sid-2*, an intestinal luminal transmembrane protein. Subsequent systemic spread of the dsRNA throughout the insect body is thought to involve other processes as it does in *C. elegans*.

Once the long dsRNA is inside the cell, it is cleaved into small interfering RNAs (siRNAs) of 21–23 nt in length by the ribonuclease III enzyme called DICER. With the assistance of dsRNA-binding motif protein (dsRBM), these siRNA are unwound and one strand, known as the guide strand, is preferentially loaded onto a multi-subunit protein complex termed the RNA-induced silencing complex (RISC), while the passenger strand is degraded (Winter et al., 2009, Burand and Hunter 2013, Baum et al., 2007). RISC is an siRNA-directed endonuclease that catalyzes the cleavage of a single

phosphodiester bond on target ssRNA molecules that have a sequence that is complimentary to that of the guide strand. Careful selection of siRNA sequences that favor incorporation of the antisense strand into RISC may improve efficiency and specificity of RNAi (Aronstein et al., 2011). An essential member of the RISC complex is the RNase H enzyme Argonaute (AGO) which mediates recognition of the target mRNA. The RISC is directed by the siRNA guide strand to locate mRNA containing specific nucleotide sequences that are complementary to the guide, it then binds and cleaves the homologous mRNA, which consequently leads to the latter's degradation. This blocks translation of the mRNA target, making it non-functional and the genes 'silenced' (Sharp, 2001).

The RNAi process has been described as 'post-translational gene silencing' in plants and as 'quelling' in fungi (Fulci et al., 2007). Characterization of this process as a ubiquitous strategy of gene silencing in eukaryotes has opened the door for new areas of research in a broad area of biotechnology from medical science to insect pest control with its high specificity for a specific nucleic acid sequence offers the possibility of being a species-specific pest control (Borovsky et al., 2005). RNAi has been recently applied in the field of entomology as a method of pest control by delivering dsRNA with a sequence that matches that of an essential gene of the target insect to induce a gene knockdown effect of this essential gene that results in insect mortality. dsRNA silencing can induce morbidity in the insects if there is efficient uptake of the dsRNA (Baum et al., 2007).

Micro-injection of dsRNA into adults and late instars has been used to silence genes in *Tribolium castaneum* (Herbst 1797) and similar experiments displayed an

RNAi response in the lepidopteran *Spodoptera litura* (Fabricius 1775) when injected with dsRNA (Baum et al. 2007). However, application of dsRNA through injection is not practical as a strategy of pest insect control. One strategy for efficient control would be through oral uptake of the dsRNA (Zhou et al., 2008). Baum et al. (2007) provided evidence for potential use of RNAi to control insect pests through the use of transgenic plants producing a hairpin dsRNA construct against specific insect target genes. Several research groups have explored oral uptake of dsRNA as a gene-silencing strategy in insects among seven orders. A broad spectrum of insects, from agricultural to medical pests, has been studied in dsRNA feeding trials (Zhou et al., 2008, Eleftherianos et al., 2006, Mutti et al., 2006, Hammond et al., 2000). The first report of oral delivery of dsRNA to initiate RNAi in an insect involved the light brown apple moth, *Epiphyas postvittana* (Walker 1863), which is a pest of horticultural crops in Australia and New Zealand; experiments demonstrated the down-regulation of a gut carboxylesterase (EposCXE1) and a pheromone binding protein (EposPBP1) after oral delivery of dsRNA solutions (Turner et al., 2006).

The target gene chosen for the purpose of the research presented in this thesis is a propriety gene expressed in the midgut of the ACP. This tissue in the midgut was chosen as the target because it is one of the first tissues of the insect that come in contact with ingested dsRNAs and therefore we assume that this tissue will come in contact with the highest concentration of dsRNA within its cytoplasm. Also, the midgut is a region of the gut that has the highest metabolic activity and is a source of many digestive processes that are essential for nutrient uptake. The midgut consists of a single layer of columnar cells with microvilli, endocrine cells, and stem cells at the base.

The midgut is designed to absorb nutrients from the gut lumen with a large absorption area with many channels and endocytosis apparatus (Hakim et al., 2010).

RNA-dependent RNA polymerase (RdRp) is an enzyme, which catalyzes the replication of an RNA template and is responsible for amplification to other cells. Insects lack an endogenous RNA-dependent RNA polymerase (RdRp), which means the dsRNAs are unlikely to be amplified using this method (Price and Gatehouse, 2008). In order for RNAi to be effective the dsRNA must be directly administered to the cells and tissues where the suppression of the target gene is to take place (Burand and Hunter, 2013). In the case of my research, ACP is expected to take up dsRNA orally while feeding on the phloem.

In order for the RNAi response to be initiated, a dsRNA fragment must be produced and available for uptake by the cells of ACP. This can be done using transgenic plants, Microinjection of hairpin dsRNA or a para-transgenesis using a viral expression vector as a surrogate to transport the dsRNA fragments containing ACP essential target genes (TG). In this research a para-transgenic plant virus would be used to deliver the dsRNA by oral uptake from the phloem to the insect midgut while feeding.

Citrus Tristeza Virus

The expression vector that was used to engineer citrus to produce dsRNA molecules within the phloem sieve elements was developed from the citrus tristeza virus (CTV) (Satyanarayana et al., 2001). CTV is a common virus in citrus plants and exists as a collection of a multitude of strains, each with different degrees of virulence (Cervik et al., 1996). CTV is found in most of the major citrus-producing regions of the world (Hilf et al., 1999). CTV belongs to a distinct closterovirus lineage within the alpha-like

superfamily of positive strand RNA viruses (Bar- Joseph, 1989, Karasev et al., 1994). Some CTV strains are essentially symptomless while others induce symptoms of citrus death and decline (Karasev et al., 1999), especially when the citrus scion is grafted to a sour orange rootstock. Symptoms include stem pitting, stunting, reduced yield and leaf yellowing (Ayllon et al., 2001). The strain labeled T30 was used as the genetic source to construct a virus based expression vector for citrus because this strain is non pathogenic while still infectious. It is considered a mild strain because it is essentially symptomless (Hilf et al., 1999).

CTV forms a flexible filamentous viron (Bar-Joseph et al., 1985, 1989) with two capsid proteins of 25 and 27 kDA, each coating about 95 and 5% of the particle length, respectively, and a coat protein with a molecular weight of 25kDa (Karasev et al., 1994). The genome of CTV is a single, messenger sense RNA of 19,296 nucleotides, making CTV the largest known ssRNA plant virus (Hilf et al., 1995).

The genome encodes 12 open reading frames (ORFs), potentially coding at least 17 protein products (Ayllon et al., 2001). ORFs 2 to 11, located in the 3' moiety of the genomic RNA are translated primarily by sub genomic RNAs. A unique transcription method assumed to stably maintain and express their unusually large, single component RNA genomes is in the form of sub-genomic RNAs (sgRNAs) (Navas-Castillo et al., 1997). The sub genomic fragments were analyzed by Northern blot hybridization from biologically diverse CTV isolates by Hilf et al. in 1995 to reveal the presence of potential sub-genomic RNA's that were dsRNA. In CTV infected plants, a set of dsRNA "replicative intermediate forms" is present (Hilf et al., 1995, Flores et al.,

1999). Sub genomic RNAs are present in infected cells, but are not encapsidated (Dolja et al., 1994).

The complete nucleotide sequence of the genomic RNA of CTV isolates T36, which is a infectious and disease causing strain; and T30, a mild isolate, which is infectious but non pathogenic, from Florida have been determined and have identical genome organization, but variable nucleotide divergence (Ayllon et al., 2001).

CTV is aphid-transmitted semi-persistently and has a very narrow host range confined to species of Rutaceae, in which it is limited to the phloem-associated cells (Karasev et al., 1994). In infected plants, long viruses are located primarily in the phloem, where they form particle aggregates (Dolja et al., 1994). CTV is transmitted by four species of aphids, but in Florida it is transmitted by *Aphis gossypii* Glover, the cotton aphid, and *Toxoptera citricida* Kirkaldy, the brown citrus aphid (Gottwald et al., 2007). The aphid retains the infectious virus for 1-3 days (Dolja et al., 1994). Although aphids spread the disease naturally, infections are also perpetuated in commercial citrus plantings through vegetative propagation (Hilf et al., 1999)

The CTV Genome and Gene Cloning

ORF 1a encodes a 349kDa polyprotein containing two papain-like protease (PRO) domains plus methyltransferase (MT) like and helicase (HEL) like domains. Translation of the polyprotein is thought to continue through the polymerase domain (ORF 1a) by a + 1 frameshift. ORF 1a and 1b are all that is required for replication in protoplasts (Satyanarayana et al., 1999). Ten 3' ORFs are expressed by sg mRNAs (Hilf et al., 1995). The two coat proteins (CP), HSP70 homolog and p61 are required for efficient virion assembly and also necessary for passage of the virus from protoplast to protoplast in order to amplify inoculums for infection of citrus trees (Satyanarayana et

al., 1999). The p6, p20 and p23 proteins are needed for the infection of plants, and along with CP, p20 and p23 are suppressors of RNA silencing (Lu et al., 2004). The genome and ORFs are in a linear construct (Figure 4-6)

CTV produces 10 3' coterminial sgRNAs (both ss and ds RNA) that function as mRNAs for the ten 3' genes (Hilf et al., 1995). The general rule that applies is that the genes located nearer the 3' terminus are usually expressed at the highest levels, thus p20 and p23 genes have the highest amount of sgRNAs, The inserted gene and the expected abundance of subgenomic fragments relative to the 3' end (Figure 2-4). Transgenic CTV will naturally make dsRNA intermediate replicates in the phloem of the plant, which can to initiate an RNAi response in ACP when it feeds on dsRNA containing gene fragments of their own essential genes.

Separate constructs were produced: one containing the psyllid target genes (TG); and one containing a sequence encoding the entire Green Fluorescent Protein (GFP). The constructs were produced by inserting these sequences into the CTV genome, near the 3' end (see Figure 2-7). The recombinant CTV sequence was then inoculated into citrus plants by Dr. Siddarama Gowda and in the laboratory of Dr. William Dawson at the Citrus Research and Education Center in Lake Alfred, Florida. These sequences were placed under the direction of a CTV CP controller element (CE) then the whole construct was cloned into a plant expression vector, pCAMBIA1380.

pCAMBIA1380 is an *Agrobacterium tumefaciens* vector used for the production of transgenic plants (Figure 2-8). It contains a kanamycin resistance gene for bacterial selection and a hygromycin B resistance gene for plant selections. Once the TG or GFP sequences are transformed into pCAMBIA1380 they were agroinfiltrated into mesophyll

protoplast cells of *Nicotiana benthamiana* (*N. benthamiana*). Protoplasts are plant cells which have the cell walls completely removed and are therefore widely used for DNA transformation since the cell wall can inhibit the passage of DNA into the cell.

Protoplasts can be regenerated into whole plants through use of plant growth regulators that stimulate growing protoplasts to regeneration shoots. This allowed the transformed plasmid to be amplified in *N. benthamiana* by successive passages of these protoplasts. Virions were then isolated and partially purified through a sucrose cushion and Citrus Macrophylla citrus trees were inoculated, after 2 -3 weeks the plants were trimmed and new flush was allowed to develop.

In preliminary experiments a Green fluorescent protein (GFP) sequence was inserted into the genome of CTV, again by Dr. Siddrame Gowda at CREC, and then inoculated into *C. Mac* citrus. GFP comes from jellyfish and characteristically fluoresces in the presence of ultra-violet (UV) light, so in this case CTV/GFP infected citrus (Figure 2-9) were analyzed under a UV light which creates a fluorescence consistent with GFP in the bark peel of infected plants (Figure 2-10). A closer look was taken at the veins of leaves (Figure 2-11) and also directly at the phloem (Figure 2-12). This determines the presence of GFP while also confirming that gene sequences can successfully be incorporated into the genome of CTV and are able to replicate and produce proteins in the phloem of infected citrus trees. In the CREC laboratory these sequences have been shown to be persistent in the trees for 8 years (Dr. W. O. Dawson, personal communication).



Figure 2-1. Symptoms of HLB cause characteristic yellowing of the leaves and veins of infected citrus. (Photo by University of Florida)



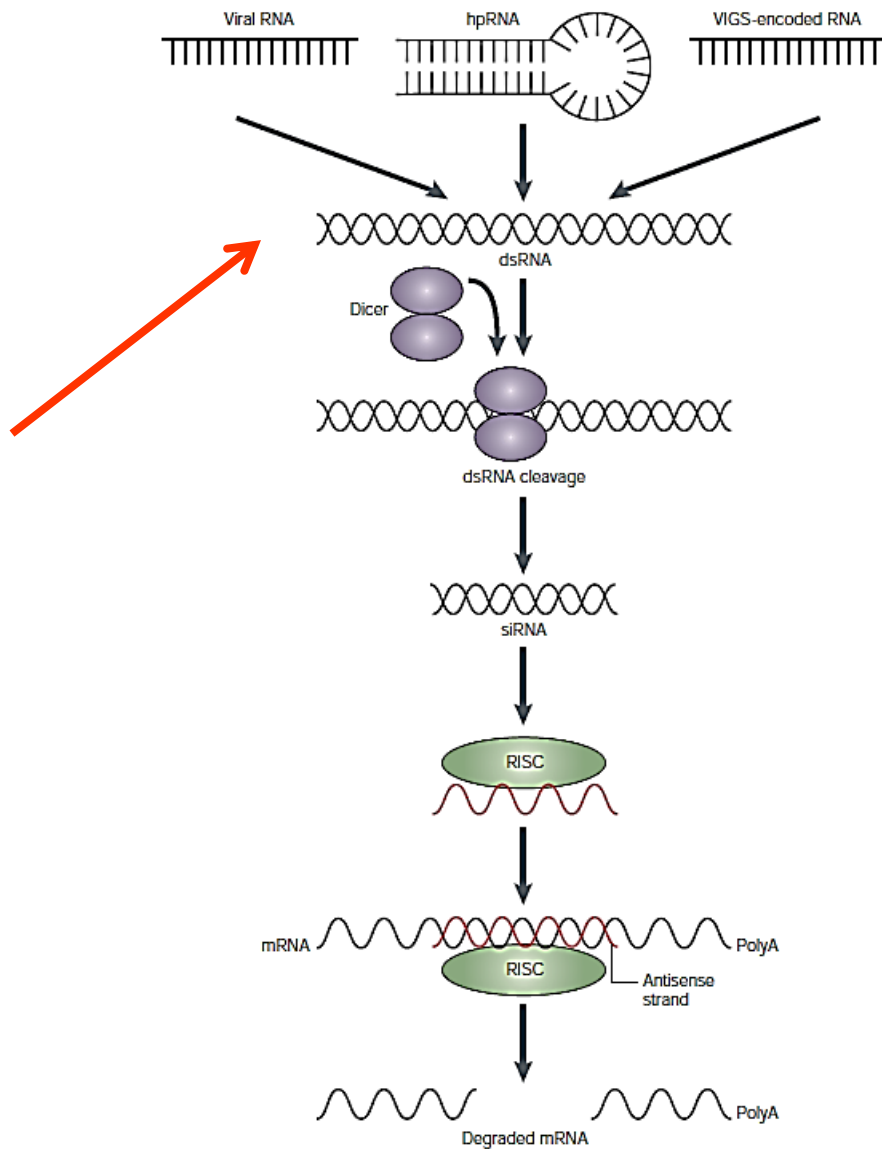
Figure 2-2. Fruits produced by plants infected with HLB are characteristically unripe with a bitter taste (Photo by USDA-ARS)



Figure 2-3. ACP is responsible for transmission of HLB, adults characteristically feed on the phloem of citrus (Photo by USDA-ARS)



Figure 2-4. Five instars of the Asian citrus psyllid (Photo by USDA-ARS)



Waterhouse and Helliwell, 2002

Figure 2-5 Diagrammatic representation of the RNAi pathway inside a cell first initiated by fragments that can form a dsRNA fragment, this dsRNA fragment is cleaved and complementary sequences are degraded to prevent foreign mRNA sequences being made. Source: Waterhouse and Helliwell (2002)

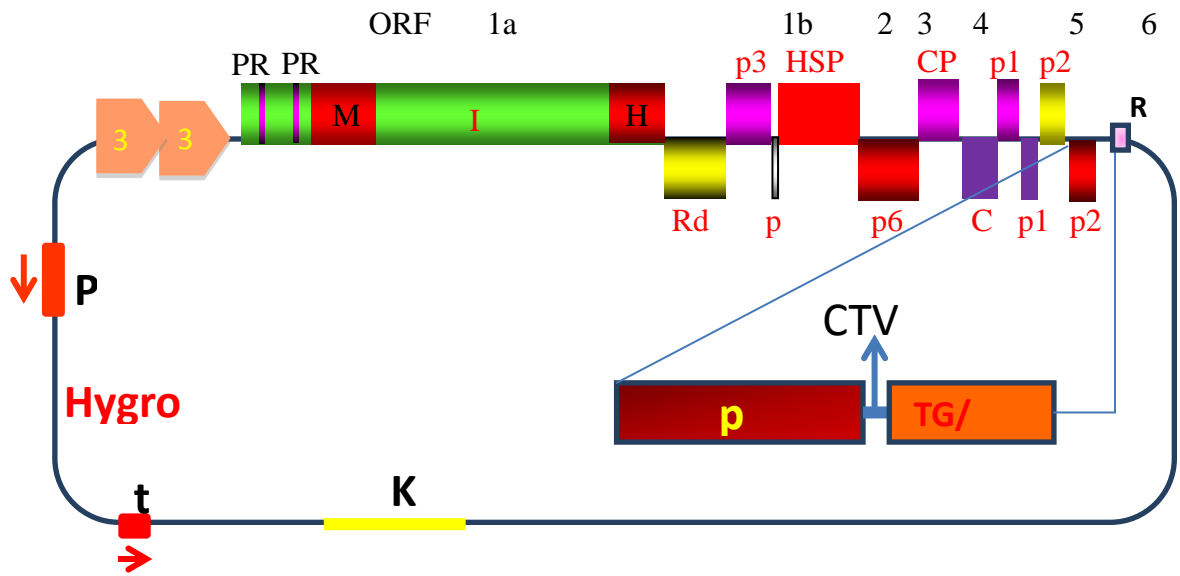


Figure 2-8. CTV in binary vector pCAMBIA 1380 containing TG/GFP in the 3' end



Figure 2-9. Plant inoculated with CTV/GFP still shows the presence and persistence of the GFP sequence 8 years after in citrus (Photo by USDA-ARS)



Figure 2-10. Bark peel of *C. Mac citrus* inoculated with CTV with an insert of GFP, fluorescing in the presence of ultra-violet light (Photo by USDA-ARS)

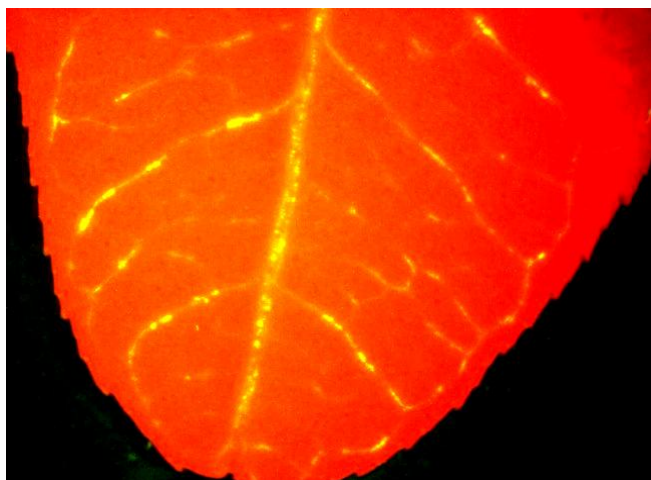


Figure 2-11. Leaf vein tissue under a UV light detecting the presence of GFP in citrus inoculated with CTV/GFP (Photo by USDA-ARS)

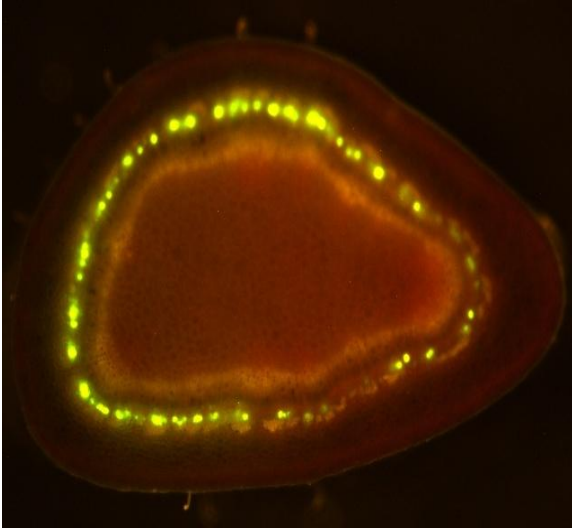


Figure 2-12. Phloem tissue of citrus infected with CTV/GFP fluorescing under UV light, showing the presence of GFP (Photo by USDA-ARS)

CHAPTER 3 CHARACTERIZING AND ANALYZING RNA FROM TRANSGENIC CTV

The citrus industry in Florida is searching for sustainable prevention for the spread of citrus greening disease, or Huanglongbing (HLB), a serious disease of citrus worldwide that ultimately leads to the death of citrus. Florida, being the largest citrus producer in the USA, needs a sustainable control mechanism to save the \$8.91 billion agricultural economy. With no cure for the disease, efforts in current control strategies have focused on controlling the Asian citrus psyllid (ACP), *Diaphorina citri*, the insect vector of HLB.

My research focuses on the emerging molecular technology of using RNA interference (RNAi) to potentially control ACP. RNAi is a naturally occurring cellular defense mechanism that occurs in all eukaryotic organisms, which targets invading foreign, double-stranded RNA (dsRNA) molecules such as viruses. Studies have shown that feeding ACP dsRNA containing sequences that match specific and essential gene coding regions will consequently lead to these genes being down-regulated and cause mortality in the insect (Hunter et al., 2012).

Citrus tristeza virus is a naturally occurring phloem-limited virus occurring in citrus. In my research, it is used as an expression vector to produce dsRNA that have sequences matching the ACP's own genes. CTV is a ssRNA virus that naturally produces dsRNA fragments in the form of replicative intermediates in the phloem. CTV was engineered to contain sequences that match an ACP gene, Target Gene 1 (TG1) [the true name of this gene is protected for proprietary reasons]. The dsRNA sequences are considered toxic when ingested by ACP.

In this chapter, the RNA fragments made by the CTV are characterized and their abundance in plants is quantified.

Materials and Methods

Citrus macrophylla infected with transgenic CTV were brought from the University of Florida Citrus Research and Education Center in Lake Alfred, FL. The plants were transported to a temperature and humidity controlled greenhouse at the USDA-ARS Horticultural Research Laboratory in Fort Pierce, FL. Treatment plants contained transgenic CTV with TG1 or CTV with green fluorescent protein (GFP) which is not toxic to ACP. Six cuttings were made from one of the CTV/TG1 plants and four cuttings were made from two CTV/GFP plants.

Four to six leaves weighing approximately 0.8 mg each were picked from a plant cutting. All leaves of the same treatment were immediately put into labeled plastic re-sealable sample bags and placed in liquid nitrogen. Leaves taken from liquid nitrogen were immediately ground with a mortar and pestle to a fine powder; liquid nitrogen was poured into the mortar if the leaves needed to be ground to a finer powder. The powder was then stored at -80°C until used.

Different Development Stages of Plant Cuttings

Four stages of flush development were taken from four growth points of the CTV/TG1 plants in order to analyze TG1 abundance in each. All four stages were taken from the same plant and were selected according to the plant that displayed all four stages. Stage 1 was the youngest flush of the leaves (Figure 3-1), stage 2 was the youngest small leaves (Figure 3-2), stage 3 was larger, tougher leaves (Figure 3-3), and stage 4 was the oldest, mature leaves (Figure 3-4). Six replications of each

developmental stage, taken from different cuttings, were grouped and extracted together according to developmental stage.

At room temperature, 4.5 g of pulverized plant tissue was added to 1 mL of TRI reagent. Six samples of each treatment were placed in a 2mL tube with glass beads and homogenized by the MP Fast Prep-24 Tissue and Cell Homogenizer (MP Biomedicals, Solon, Ohio) at 6.0 m/sec, for 60 sec, then left to incubate for 5 min at room temperature (18-25°C). Subsequently, 200 µL of chloroform were added to each, the samples were vortexed using the Vortex Gene 2 (VWR Scientific, Radnor, PA), then incubated at room temperature for 10 min and centrifuged at 12,000 rpm for 10 min at 4°C. The aqueous phase was transferred to a fresh tube, and 500 µL of isopropanol was added. The sample was then incubated at room temperature for 10 min and centrifuged at 12,000 rpm for 10 min at 4°C. The supernatant was discarded, and 1 mL of 75% ethanol (ETOH) per 1 ml of TRI reagent was added to the pellet. The samples were again centrifuged at 8,000 rpm for 8 min at 4°C. The pellet was air dried for 10-12 min and dissolved in 80 µl of nuclease-free water. This RNA sample was stored at -80°C until used.

To remove any possible DNA from the sample, RQ1 DNase, (Promega Biotech, Madison, WI) was used according to the manufacturer's protocol. Incubation was performed at 37°C for 1.25 hr. Digested RNA was extracted with an equal volume of 300 µl of low pH phenol (4.3 pH), vortexed, and centrifuged at 12,000 rpm for 10 min at 4°C. The aqueous phase was extracted with an equal volume of phenol/chloroform (1:1) added to it, vortexed, and centrifuged at 12,000 rpm for 10 min at 4°C. RNA in the aqueous phase was precipitated with 0.3 M sodium acetate (NaOAc) pH 5.2 and 2.5

volumes of 100% ETOH and maintained at -20°C overnight to allow the RNA to precipitate. The RNA was collected in a pellet by centrifugation at 15,000 rpm at 4°C for 15 min and the supernatant discarded. To the pellet were added 300 µl of 70% ice cold ETOH and the solution was centrifuged at 15,000 rpm for 10 min and air dried for 10-12 min. The RNA was re-suspended in nuclease- free water and stored at -80°C.

RNA quantity and quality were measured spectrophotometrically by calculating the 260:280 nm absorbance ratio using the Nano Drop 8000 UV-Vis Spectrophotometer (Thermo Scientific, Waltham, MA). All six samples were evaluated at one time, with the machinery being cleaned with nuclease-free water between each sample set.

Quantitative Reverse Transcriptase Polymerase Chain Reaction

Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) was used to analyze the amount of expression of a specified gene. Citrus Actin is a general housekeeping gene present in all citrus plants. Therefore, for all qRT-PCR experiments, Actin was used as a positive control. Master mixes used for qRT-PCR are given (Table 3-1). These reactions were performed using the Quanti Tect SYBR GREEN PCR Kit (Product 204143, Quigen, Hilden, Germany). Reactions were performed with Corbet Robotics CAS1200 automated liquid handling robot (Corbett Life Sciences, San Francisco, CA). Samples with no template were used as a negative control to ensure there was no contamination in the machine. All reactions were run in triplicate.

After the reactions were mixed in the Corbett robotics handling system, PCR was run on samples using the RotoGene 6000 (Quiagen, San Francisco, CA). Temperature profile was custom set 5°C lower than the melt temperature of primers used (depending on which gene was being analyzed). The temperature profiles used are shown in Table

3.2

The melt ramp was set to run from 55°C to 90°C, increasing by 0.2° each step. It was set to wait for 90 sec of pre-melt conditioning on the first step then wait 5 sec for each step afterward. Samples were stored at -20°C after the run. Results were analyzed on the RotoGene program software. Agarose gel electrophoresis (Sambrook and Russell, 2001) was performed to determine the size of the RNA fragment to ensure it was the correct amplification.

Polymerase Chain Reaction

Traditional PCR with GoTaq Green Master Mix (Promega Biotech, Madison, WI) (Table 3.3) was carried out to amplify a sequence of DNA. The reactions were manually made then put into a Peltier Thermal Cycler – 100 (PTC-100), with parameters set (Table 3-4 and Table 3-5). Once the cycle was complete, the temperature was set to stay at 4°C to minimize the polymerase activity until permanently stored at -80°C.

Agarose gel electrophoresis (Sambrook and Russell 2001) was performed to separate and isolate the amplified fragment of DNA. Nucleospin Gel and PCR Clean Up Kit (Macherey-Nagel, Duren, Germany) was used according to manufacturer's instructions to clean the DNA. Cloning into a plasmid was carried out using the TA Cloning Kit with (pCR 2.1 Vector) (Life Technologies, Grand Island, NY) according to manufacturer's instruction.

RNA Digest

The digestion of RNA was carried out to ensure that the RNA fragments made by the CTV were dsRNA. The digest will get rid of all the ssRNA and just leave the dsRNA. RNase buffer was made with 5 nM of ethylenediaminetetraacetic acid (EDTA), 300 nM sodium chloride (NaCl), and 10 nM Tris- HCl, at pH 7.5. Samples of RNA were first diluted to 100 ng/μl then 300 μl of the buffer was added to 80 μl of RNA sample. Before

incubation at 37°C, 50 µl of the sample were removed and put on ice. This was the 0 time interval for incubation (no incubation). To the rest of the RNA sample and buffer, 0.02 ng/µl of RNase A (Invitrogen, Grand Island, NY) was added then incubated at 37°C. Aliquots of 50 µl were taken at time intervals of 5, 10, 15, 30, and 45 min after 0 time incubation and immediately put onto ice. Samples were stored at -80°C until ready to run on qRT-PCR.

Plates for Cultivating Bacteria

Plates were made using LB Agar Media (Fisher Scientific, Waltham, MA), a nutritionally rich medium for the cultivation of *Escherichia coli* strains. To make 10 plates, 200ml of water and 8 g of LB media with agar were mixed using stirring magnets for 10 min in a 200ml glass beaker with a plastic twist cap. The beaker with media was placed in an autoclave set to L3 (liquid media), making sure the cap was loose to let out steam in the process. After autoclaving, the mixture was left to cool until able to touch but not enough to solidify. To the beaker was added 200 µl of antibiotic (kanamycin 50) and the media was stirred for approximately 2 min. The mixture was poured into Petri dishes (100 mm in diameter and 15 mm in height) under a hood with the airflow on until the mixture covered the bottom surface of the Petri dish. Lids were left half off until the mixture had solidified. The plates were labeled, dated, sealed with Parafilm, and stored at 4°C until needed. Before using, 40 µl of X GAL diluted to 40 mg/ml was spread onto the media in each plate and the liquid was left to soak into the media. Growing *E.coli* colonies on the plates was achieved following the TA Cloning Kit manufacturer's protocol. Transformation of the colonies was completed following the TA Cloning Kit according to the manufacturer's instruction.

Standard Curve

Standard curves (Figure 3-5 and Figure 3-6) were calibrated for both Actin and TG1 to analyze results in terms of copy number per reaction instead of ng per reaction using a pure plasmid of each gene piece. Using Avogadro's constant ($6.02214129 \times 10^{23}$), comparative values taken from the RotoGene program were converted into copy number per reaction.

An ANOVA ($\alpha = 0.05$) was performed on the results obtained by qRT-PCR to determine if there were significant differences among different plants containing CTV/TG1 sequences. An ANOVA ($\alpha = 0.05$) was also performed on the results obtained by qRT-PCR of four developmental stages of the plant to determine differences among them. Consequently Tukey's multiple comparison test ($\alpha = 0.05$) was performed to further analyze the differences.

Results

Sequences for TG1 were detected in all plant cutting samples (Figure 3-7) and also detected in the four developmental stages 1, 2, 3, and 4 of these plants that contained the transgenic CTV construct with TG1 (Figure 3-8). The nature of these fragments was observed to be dsRNA after using RNase digest incubation then analyzing on qRT-PCR (Figure 3-9).

The standard curve for the Actin control gene was $y = 1 \times 10^{12} e^{-0.765x}$ (Figure 3-5) whereas the standard curve for TG1 was $y = 1,965,144,263,985.66 e^{-0.76x}$ (Figure 3-6). These standard curves were used to convert the comparative values taken from the Rotogene into copy number per reaction.

Copy number per reaction varied among leaves and plants containing the same CTV/TG1 construct. Plant A had the highest variation among its leaves (SEM:

1.14E+08) (Figure 3-7). Plant D contained the highest amount of the TG1 sequence (average copy number: 1.45E+09) and plant C contained the lowest (copy number: 705141029). The ANOVA showed that there were significant differences ($F = 20.85$, $df = 11$, $p = 0.0004$) in expression of the target gene among the different plants. The Tukey's post test ($\alpha = 0.05$) revealed that the expression was significantly higher in plant A versus C and D. The expression was also significantly higher in plant B versus plant C. The expression in plant C was significantly lower than in plant D.

Expression levels of the TG1 sequence were detected in all four flush developmental stages (Figure 3-8), with the highest titer in stage 2 (average copy number: 178396778.4), closely followed by stage 1 (average copy number: 113922159.4). Stages 3 and 4 had a lower titer of the TG1 sequence. Therefore, the titer of TG1 sequences is higher in the youngest flush of the plant. The ANOVA showed that there were significant differences ($F = 901.3$, $df = 11$, $p < 0.0001$) in expression of the TG1 among the four developmental stages. The Tukey's post test ($\alpha = 0.05$) revealed that the expression was significantly higher in the second stage than in the first, third, and fourth stage. The expression was also significantly higher in the second versus the third and the fourth stage.

TG1 RNA incubated with RNase showed a decline in ssRNA for actin after 5 min of incubation (Figure 3-9). TG1 RNA showed a decline after 15 min of incubation then a constant level of average copy number which is consistent with the assumption that there was no more degradation of ssRNA after 15 min. Therefore, all copy numbers came from dsRNA.

Discussion

Citrus tristeza virus (CTV) makes dsRNA fragments in the form of replicative intermediates in the phloem and companion cells of citrus (Hilf et al., 1995). When TG1 sequences are inserted into the CTV genome, the sequence are replicated and can be identified in the phloem of citrus and tissue when detected after qRT-PCR. Therefore, it is clear that CTV can vector TG1 sequences into the plant tissue of citrus. Four cuttings were made from the original CTV-infected plant received from CREC in Lake Alfred, and these four were all tested for the presence of TG1 sequences. TG1 was present in all four cuttings, which means CTV is replicating the TG1 sequences in replicative intermediates and these are being distributed throughout the phloem. Varying amounts were detected in each of the plants with some having a higher copy number than others. This is because CTV is unevenly distributed throughout the plant and leaves contain different copy numbers of the TG1 (Siddrame Gowda personal communication), resulting in variable copy numbers of the TG1.

Since there is no conclusive evidence to indicate where the highest copy numbers would be in the plants, I investigated four flush developmental stages of citrus inoculated with CTV with the TG1 insert to see if CTV was found in higher abundance in younger plant tissue. It is likely this will be an advantage in a potential pest management scenario because ACP adults most often feed on the younger leaves and flush of the plants and eggs are laid on flush where nymphs developed (Childers and Rogers 2005). It is also evident that the nymphs of ACP are the best candidates to acquire the bacteria associated with HLB. Nymphs are reliant upon the young leaves for feeding and development and this is also where eggs are laid. With high copy numbers

of the TG1 sequence effects on development would be expected when feeding plants containing CTV with the TG1 sequence.

The RNA interference (RNAi) pathway is first initiated when dsRNA comes into contact with the cell cytoplasm (Aronstein et al., 2011). The presence of the TG1 sequence in the plant cuttings and in all flush developmental stages of the citrus was verified, but it is important to know if this sequence is single-stranded or double-stranded since it would not initiate an RNAi response in ACP if it were single stranded. Creation of double-stranded fragments of TG1 by CTV was verified using an RNase enzyme digest which would degrade all ssRNA material. Actin was used as a control in this digestion experiment and would be ssRNA. After 5 min of incubation with the RNase A, Actin was no longer detected. This indicates the ssRNA of Actin was degraded by the enzyme. The RNA extracted from TG1 was also digested with the RNase A, but after 5 min of incubation it gradually decreased and decreased again after 10 min of incubation, then stabilizing in quantity after 15 min of incubation. It can be assumed that all the ssRNA forms of TG1 were eliminated by 15 min of incubation, and since they stabilized for the total incubation time of 60 min, the remaining levels can be attributed to dsRNA fragments from the CTV.



Figure 3-1. Stage 1 development (Photo by University of Florida)



Figure 3-2. Stage 2 development (Photo by University of Florida)



Figure 3-3. Stage 3 development (Photo by University of Florida)



Figure 3-4. Stage 4 development (Photo by University of Florida)

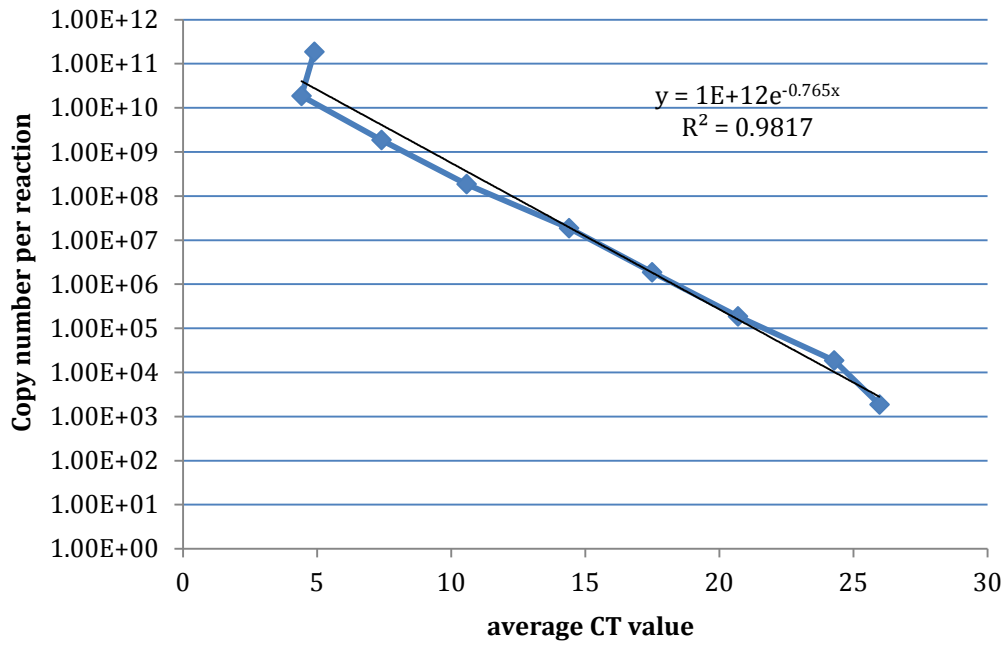


Figure 3-5. Actin calibration curve with trendline

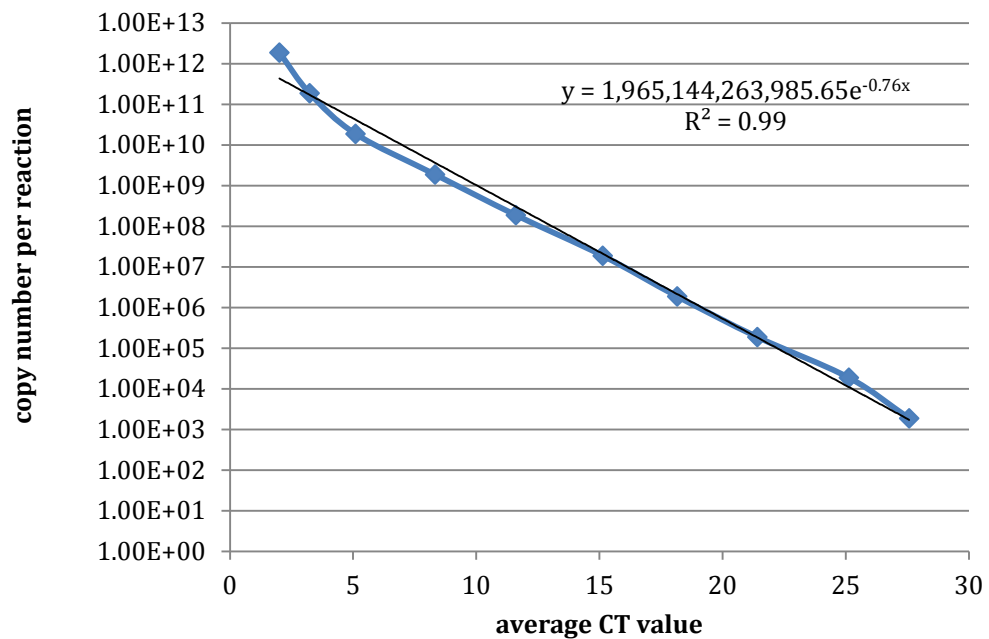


Figure 3-6. TG1 calibration curve with trendline

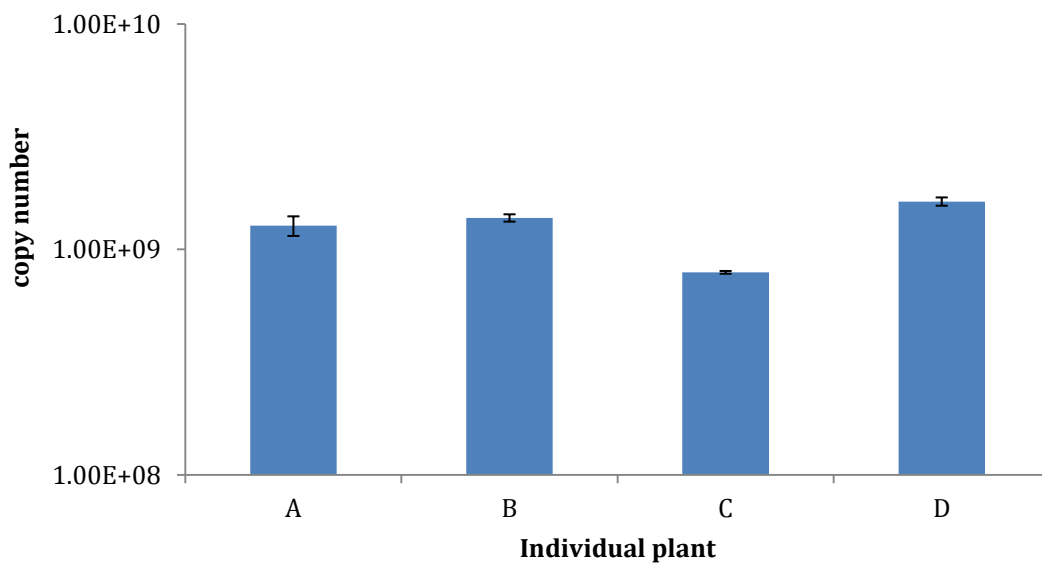


Figure 3-7. Expression levels of TG1 in four citrus plants after inoculation with CTV containing a TG1 construct.

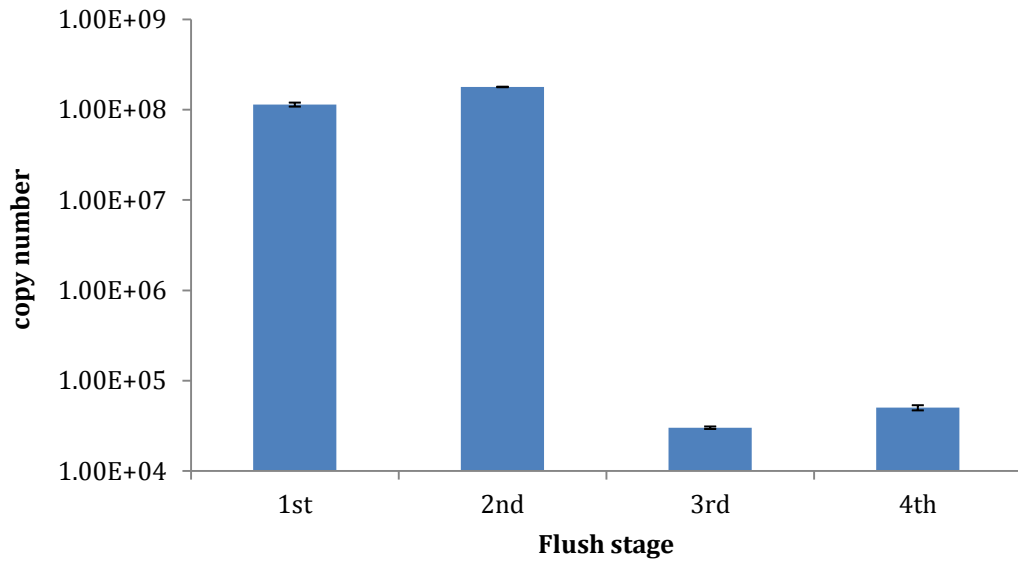


Figure 3-8. Copy number of TG1 sequence in four plant developmental stages of six different plants after inoculation of CTV with a TG1 construct.

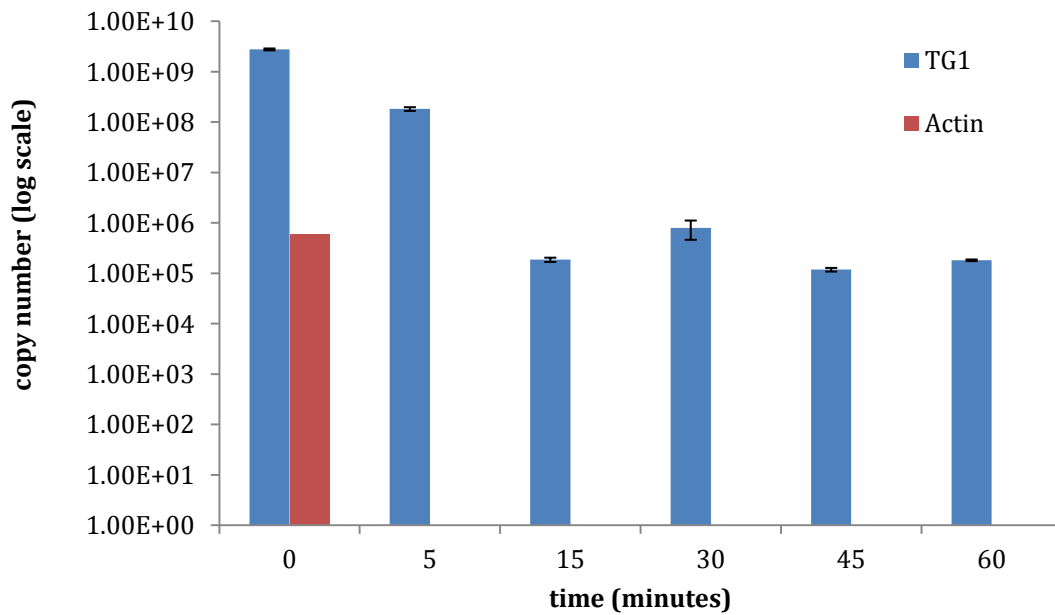


Figure 3-9. Expression level of Actin and TG1 RNA after digestion with RNase A enzyme

Table 3-1. Master mix formula for all qRT-PCR reactions in Corbett liquid handling system.

	RT (μL)	noRT (μL)
SYBR GREEN	6.25	6.25
RT	0.125	0
Nuclease-free water	0.125	0.25
Primer	1 (F and R)	1 (F and R)

Table 3-2. qRT-PCR temperature cycling run profile.

Step	Temperature (°C)	Time (mins)
Hold 1	50	30
Hold 2 (Hot start)	95	15
40 cycles:		
Denaturing	94	15
Annealing	55	30
Extension	72	30

Table 3-3. Master mix formula used for all PCR reactions

GoTaqGreen master mix	12.5
Primers (10μM stock)	2
Sample	5
Nuclease Free water	Make to volume of 25μl

Table 3-4. The initial denaturing step of all PCR reactions

Step	Temperature (°C)	Time (mins)
Denaturing	95	2

Table 3-5. Cycling profile for all PCR reactions

Step	Temperature (°C)	Time (seconds)
40 cycles:		
Denaturing	94	30
Annealing	55	30
Extension	72	30
Hold	72	5 mins

CHAPTER 4 EFFECT OF RNA ON ASIAN CITRUS PSYLLID

Asian citrus psyllid is the vector of citrus greening disease (HLB), which is a serious disease of citrus worldwide. ACP is regarded as a major pest in the citrus industry due to its ability to transmit and spread HLB (Halbert and Manjunath 2004). It is now present in Florida as well as Texas (2001) and California (2008). The ACP needs actively growing flush to lay eggs and support nymphal development. In southern Florida, population peaks of ACP occur in May, August, and October through December, with the highest levels after the first growth of flush (Bove, 2006). ACP has five nymphal instars and all life stages are associated with citrus, and the nymphs are known to acquire the bacterium associated with HLB more efficiently than the adults (Stelinski et al., 2010)

Citrus tristeza virus (CTV), a naturally occurring phloem-limited virus serves as a para-transgenic expression vector for dsRNA delivery to ACP to initiate an RNA interference response in ACP. CTV is a large, linear ssRNA virus that naturally produces dsRNA fragments in the form of replicative intermediates in the phloem. CTV was engineered to contain sequences that match an ACP gene, Target Gene 1 (TG1) [the true name of this gene is protected for proprietary reasons]. The dsRNA sequences of TG1 are toxic when ingested by ACP because as a result of the RNAi response in ACP cells the ACP's own essential gene target will be downregulated, or silenced. RNAi is a naturally occurring cellular defense mechanism that occurs in all eukaryotic organisms, which targets invading foreign, double-stranded RNA molecules such as viruses. Studies have shown that feeding ACP dsRNA containing sequences that match

their own essential genes will consequently lead to these genes being down-regulated and cause mortality in the insect (Hunter et al., 2012).

In Chapter 3, I characterized the presence of TG1 dsRNA in citrus plants inoculated with CTV with TG1 inserted. I hypothesize that the survivorship and development of ACP are significantly affected by the insect feeding on CTV/TG1-inoculated citrus. The toxicity of TG1, in the form of these dsRNA fragments, to ACP is explored through feeding assays to quantify the mortality and developmental effects on ACP.

Materials and Methods

Excised Single Leaf Assay

All adult ACP used for feeding bioassays were taken from a laboratory colony established during 2000 and maintained on healthy, uninfected citrus trees (*Citrus macrophylla*) in a greenhouse with controlled temperature and humidity at the USDA-HRL in Fort Pierce, FL. No wild ACP were introduced into this colony, and individuals from the colony are PCR-assayed every three months to ensure the colony is HLB-free (Hall et al. 2007).

Citrus plants (*C. macrophylla*) used for bioassays were infected with transgenic CTV at the University of Florida Citrus Research and Education Center (CREC) in Lake Alfred, FL. The plants were transported to a temperature and humidity controlled greenhouse at the USDA-HRL (Figure 2-1). TG1 treatment plants contained transgenic CTV with a Target Gene 1 sequence (TG1) that is toxic to ACP. GFP treatment plants contained transgenic CTV with green fluorescent protein (GFP), which is considered not toxic to ACP. Plants containing no CTV were also used as a control treatment in the experiments to verify the non-toxicity of GFP dsRNA.

Plants from CREC were re-potted in Pro-mix potting soil treated with 20/10/20 325 ppm nitrogen in a pot that carried approximately 7.78 L of soil. All plants were treated every three weeks with a 2% solution of M-PED insecticidal soap to protect them against mealy-bugs and scale insects. All plants with transgenic CTV were assayed by PCR every four months to ensure the presence of CTV with Target Gene 1 and GFP.

Approximately six plant cuttings of each CTV treatment were made from the CTV-infected plants by cutting part of a newer stem at an angle. Cuttings were then left to soak for approximately 5 min in Rootone powder diluted with approximately 10 ml of tap water. They were then placed into root cubes and their position secured by pressing the cubes to close contact of the cutting. Cuttings were left for approximately eight weeks until roots began, at which time they were taken from the root cube and placed individually into 7.78 L pots with Promix potting and left to root and grow (Figure 4-2). Cuttings were PCR-assayed every three months to ensure TG1 was still present in the form of dsRNA fragments made by the replication of CTV that infected the phloem. The purpose of the cuttings for my experiments was to provide more flush points and leaves for the experimental bioassays.

Excised Leaf Assay

Assays were prepared with feeding tubes as described by Ammar et al. (2013) with minor adjustments. Ten mature leaves were taken at random from four cuttings of each of the three treatments CTV/TG1, CTV/GFP, and no CTV. All leaves were washed thoroughly with deionized water to remove any remaining insecticidal soap and air-dried before using in experiments. The terminal base of the leaf petiole was cut diagonally with a sharp, unused razor blade to allow a wider cut area for water absorption. One

leaf petiole was placed into a 2-ml microcentrifuge tube filled with tap water. A parafilm membrane (Pechiney Plastic Packaging Company, Chicago, IL) was wrapped around the top of the tube and the petiole to secure the latter's position, to reduce water evaporation, and to prevent ACP coming into contact with the water. The excised leaf in the microcentrifuge tube was then placed into a 50-ml polypropylene feeding tube (Thermo Fisher Scientific, Waltham, MA) (Figure 4-3). Ten feeding tubes per treatment were prepared.

Adult ACP that were three weeks old were collected into separate tubes (10 adults per tube) and left at -4°C for approximately 10 min to reduce movement when transferring them to the feeding tubes. Ten adults were added to each feeding tube. Once the adults were added, a flip screw top (BD Falcon Conical Tubes with Flip top cap, BD Biosciences, San Jose, CA) was adjusted to the tube. The flip portion of the cap was removed, and a fine piece of mesh screen cloth, which provided ventilation, was put under the screw cap (Figure 4-3).

The feeding tubes were placed in a tube rack (Figure 4-3) and kept on the bench top in the laboratory (approximately 23°C) under a 60W natural light to keep ACP actively feeding. Adults were left to feed for seven days, and mortality was recorded daily. After the final recording of a trial conducted in May 2012, the ACP and leaves were stored at -80°C for PCR analysis to measure the abundance of TG1 and GFP.

Excised Flush Assay

Sections of each treatment's plants containing new flush (Figure 4-4) were selected for feeding of adults and potential oviposition. The stem of the flush was cut diagonally with an unused razor blade to allow a wider cut area for water absorption. The flush was washed thoroughly with deionized water to remove any insecticidal soap

then air-dried before use. It was then immediately placed into a 10-ml tube containing agar to prevent the plants from becoming desiccated. The agar solution was prepared beforehand with Agar Powder – Plant Tissue Culture Tested (PhytoTechnology Agar Product #A296) and 15 ml of water soluble fertilizer (Peters Professional Plant Starter Product #91140) diluted into 3.5 L of tap water. Into a 2-L, thick-walled beaker containing a magnet stir bar, 750 ml of fertilizer water were added to 4 g of the agar. A heated stir plate was used to stir the mixture and boil the agar until it became clear. Once clear, the mixture was put in the autoclave on the liquid 3 cycling setting. After being autoclaved, the agar solution was dispensed into the tubes.

A Parafilm membrane was wrapped around the top of the tube and the stem to secure the flush in place, to reduce water evaporation, and to prevent ACP coming into contact with the agar. The flush was then enclosed within a Petri dish, which had a mesh hole cut into one of the sides of the dish and a notch to allow the tube with agar to be placed so the stem and flush could pass through. Ten 3-week-old ACP (5 males and 5 females) were collected and put into each flush chamber. Once the ACP were added, the Petri-dish was sealed with Parafilm wrapped around the two plates to hold them together, to prevent ACP escaping from the feeding chamber, and to also hold the plates to the tube containing the flush and the agar (Figure 4-5) Ten replicates were prepared for each treatment (Figure 4-5).

Feeding chambers were placed in a tube rack (Figure 2-6) and kept on the bench top of the laboratory (approximately 23°C) under a 60W natural light to encourage ACP activity. Chambers were checked daily for 14 days to record mortality of adult and nymphal development. Number of eggs was counted under a light microscope and

recorded 4 days after placing the adults in the feeding chamber. At the end of 14 days, the ACP and flush were stored at -80°C.

An ANOVA was performed to compare means among treatments. A t-test was used to detect differences between treatment means.

Results

Total mortality was higher for adult ACP that fed on leaves with CTV/TG1 sequence (18%), compared to the mortality of adults on leaves with CTV/GFP sequence (2%) and leaves with no CTV (0%) (Figure 4-7). The variation among leaves of the TG1 treatment was large over the eight days of feeding, with some leaves of the same treatment causing up to 80% mortality while others caused 0%. The wide variation suggests different levels of toxicity of leaves causes mortality in varying amounts in the same treatment. Leaves containing CTV/GFP sequence provoked a low amount of ACP mortality but up to 20% on eleven leaf replicates. Adult ACP on control leaves suffered no mortality.

One trial of this experiment conducted in May 2012 suggests a correlation between ACP mortality and the amount of TG1 copy number. The range of mortality from feeding on five CTV/TG1 leaves ranges from 0% to 70%, whereas feeding on CTV/GFP leaves ranged from 0% to 10%; and there was no variation in mortality on leaves fed on leaves without CTV (Figure 4-8). Copy numbers of TG1 sequence in the leaves were analyzed from this trial (Figure 4-9) after the feeding assay was completed. Leaf 2 induced the highest mortality (70%), and the copy number of TG1 sequence in this leaf was the highest among all five leaves. Leaf 1 produced the second highest mortality among the five leaves, but it does not show the second highest value for copy number of the TG1 sequence. There was no mortality in leaves 4 and 5, yet these two

leaves produced a higher copy number of TG1 than leaf 1. This suggests that only a very high copy number of TG1 might induce significant ACP mortality.

ACP feeding on CTV/GFP laid the fewest number of eggs and were significantly different from the amount laid by CTV/TG1 ($p=0.007$). There was no significant difference between the number of eggs laid on no-CTV leaves and leaves with CTV/TG1 ($p= 0.35$).

Discussion

In Chapter 3, the presence of TG1 in the CTV dsRNA was quantified, after determining that the TG1 was present in high levels in all plants and all flush developmental stages of the citrus inoculated with CTV/TG1 sequence. It was important to test the effect of this dsRNA on the ACP feeding on the phloem of plants to assess the ability for it to be used for field applications. ACP feeding on citrus inoculated with CTV/TG1 sequence suffered increased mortality by ingesting the dsRNA replicative intermediates which contain fragments of TG1. Oral uptake of these dsRNA using CTV with inserted TG1 might be a possible control mechanism because it causes death of ACP feeding on infected plants in a species-specific manner. It is possible a certain copy number of TG1 must be accumulated in the plant tissue where the ACP is feeding to induce ACP mortality.

Higher ACP mortality was recorded on leaves with higher levels of TG1 expression, but the results were inconsistent.

CTV is unevenly distributed throughout the plant (W. O. Dawson and C. Powell, personal communication) so mortality might vary depending on the copy number of TG1 in the feeding site. The TG1 sequence had no effect on the number of eggs laid by ACP after feeding on leaves with CTV/TG1 for four days. There were, however, a decreased

number of eggs laid by ACP feeding on leaves with CTV/GFP. This could potentially mean that the GFP sequence in the CTV has some indirect effect on the oviposition activity of ACP. This could also be that the CTV/GFP sequence or the dsRNA alone has an indirect target effect on the ovipositional activity of ACP after feeding on dsRNA fragments in the phloem.



Figure 4-1. Plants with transgenic CTV kept at controlled environmental conditions for use in bioassays (Photo by University of Florida)



Figure 4-2. Cutting from original CTV inoculated citrus, which had been allowed to grow for several weeks at controlled conditions (Photo by University of Florida)

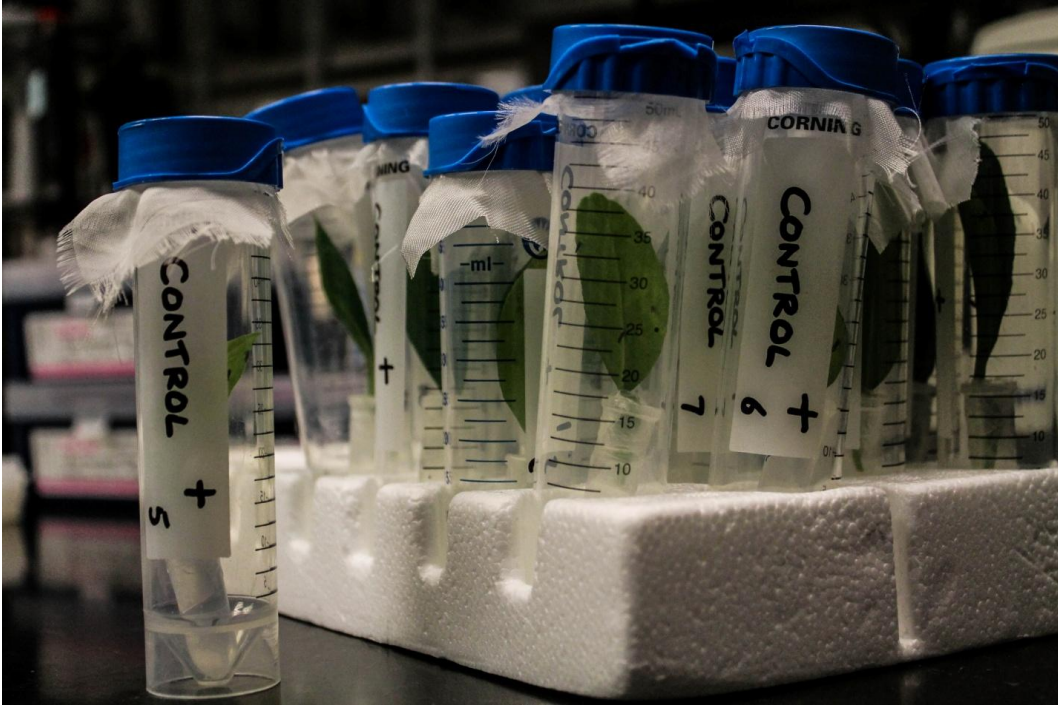


Figure 4-3. Feeding chambers for the excised leaf assay and tube rack (Photo by University of Florida)



Figure 4-4. Flush selected for excised flush assay (Photo by University of Florida)



Figure 4-5. Feeding chamber used for excised flush assay (Photo by University of Florida)



Figure 4-6. Excised flush assay in a rack on the laboratory bench (Photo by University of Florida)

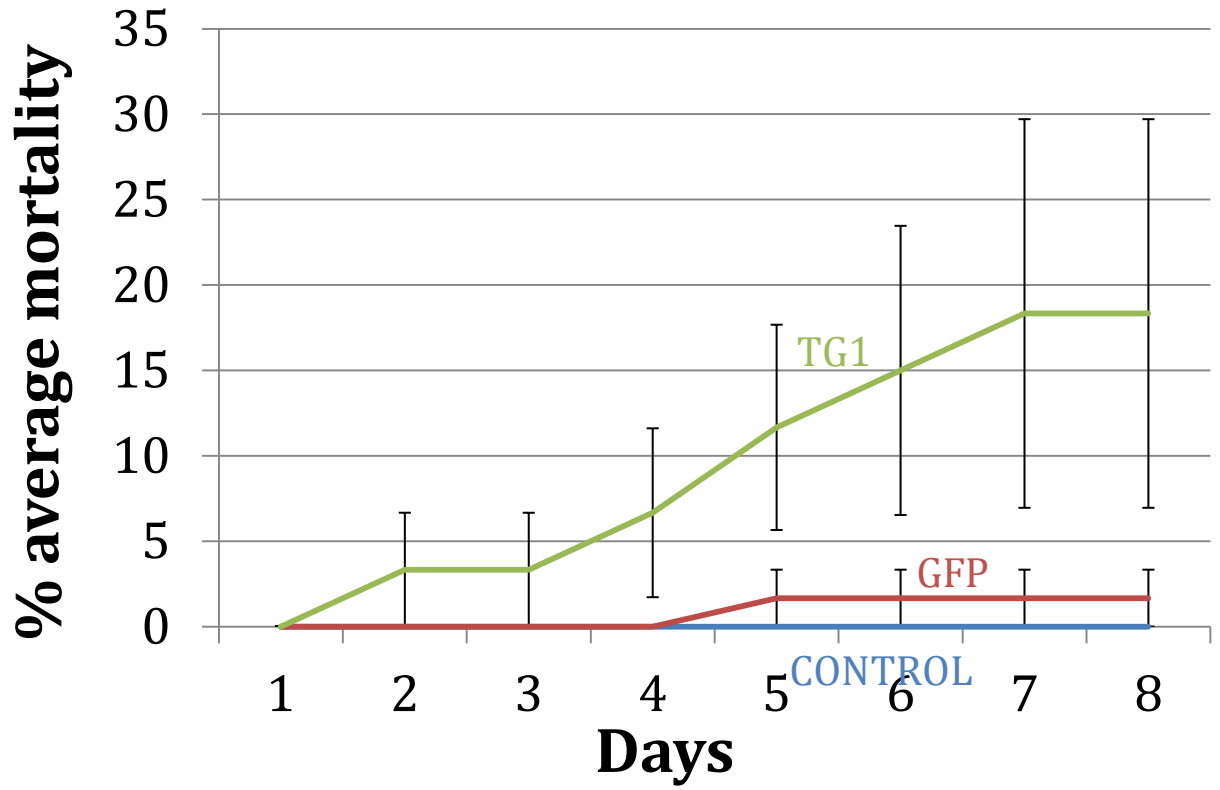


Figure 4-7. Cumulative mortality of ACP when fed on excised leaf assays from replications

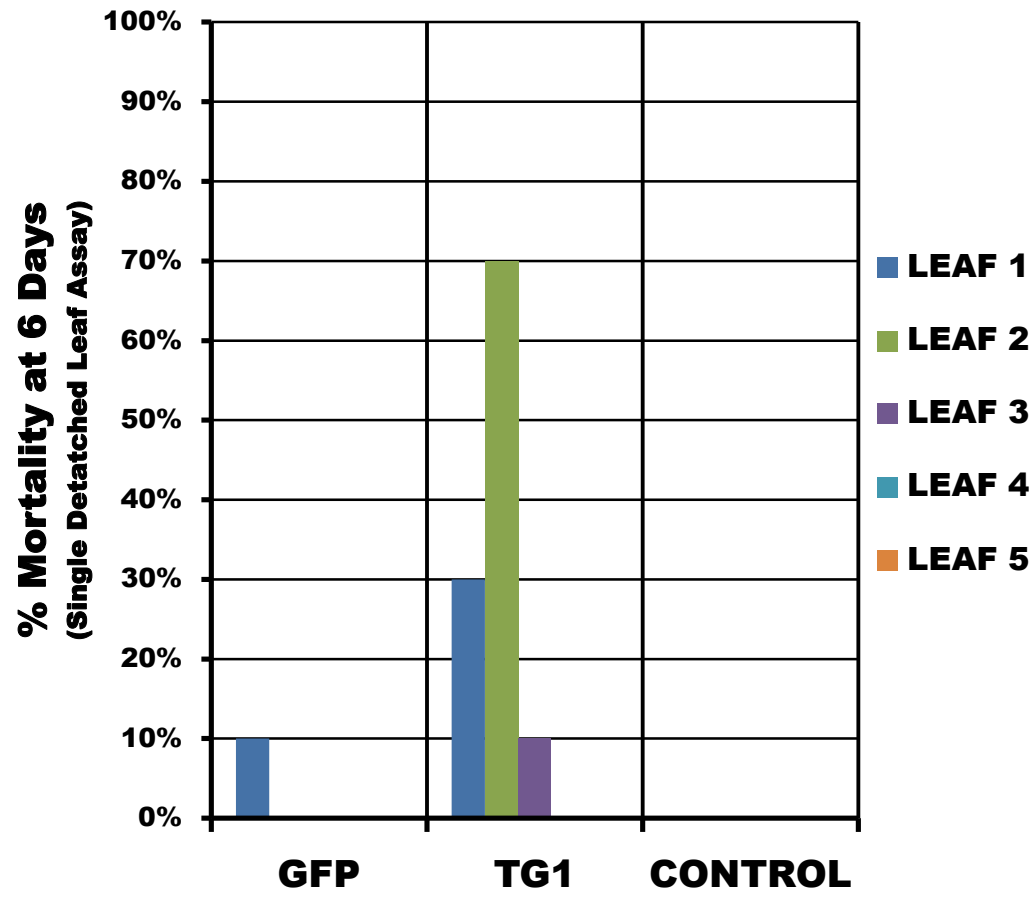


Figure 4-8. Mortality from single leaf assay in one trial.

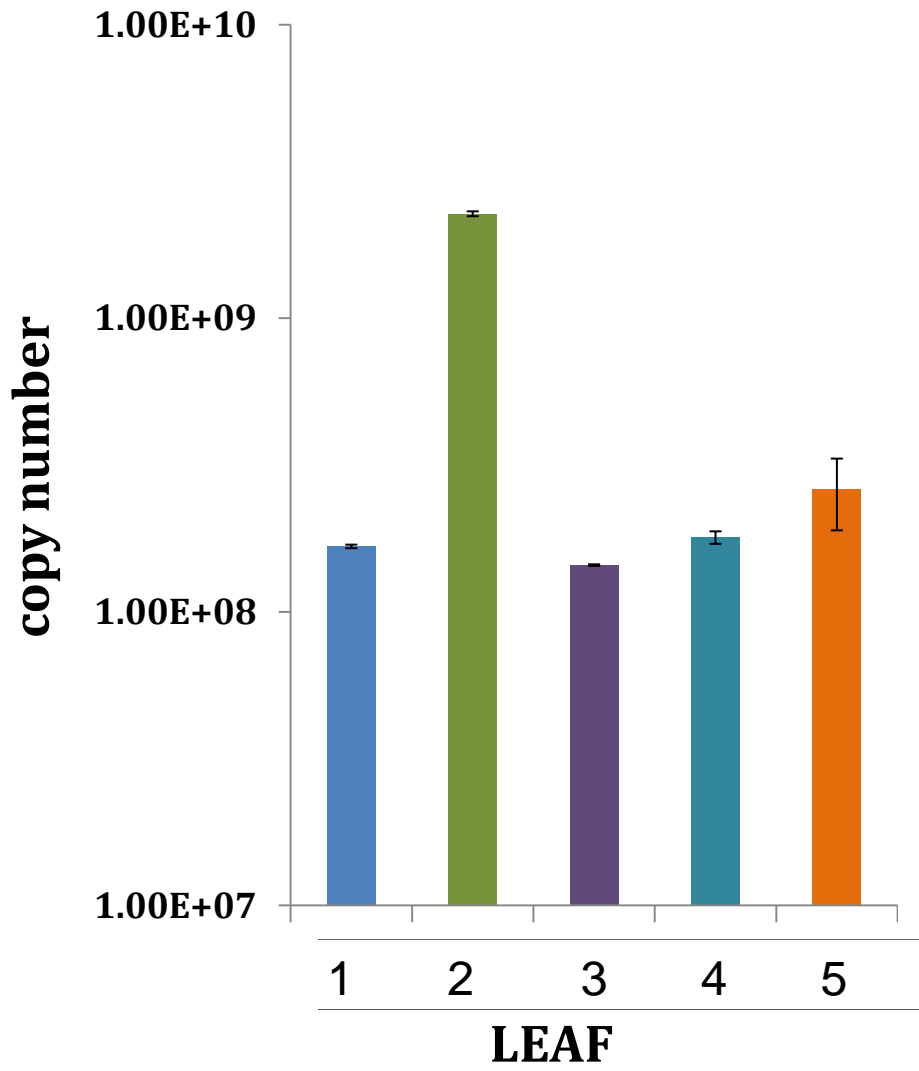


Figure 4-9. Expression of TG1 in leaves from feeding assay (Figure 4-8)

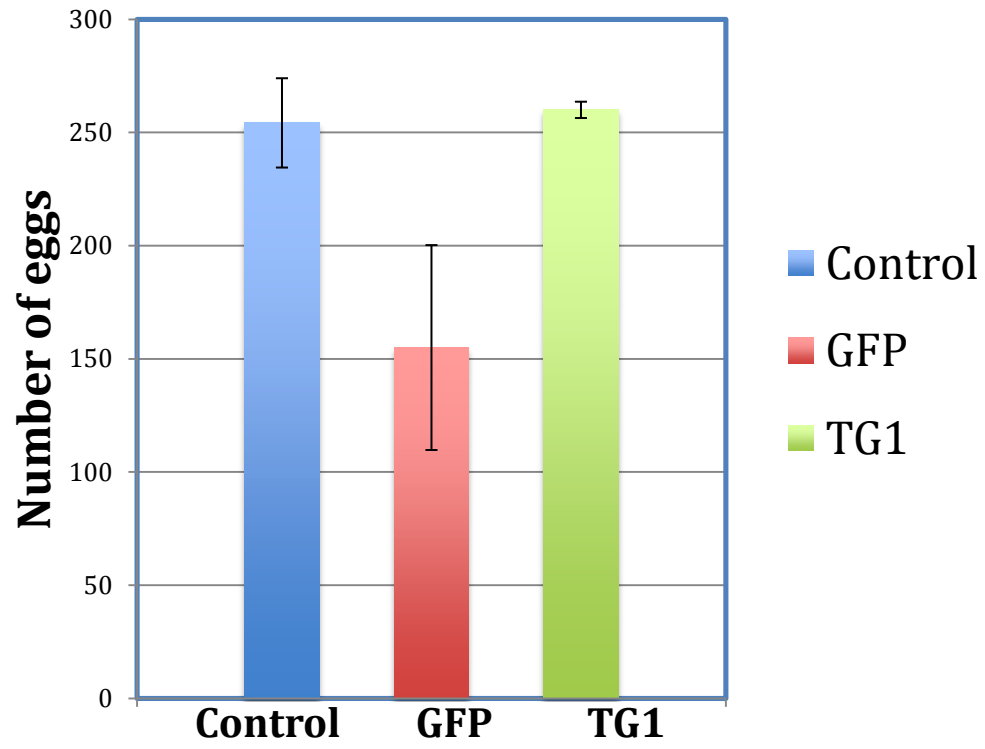


Figure 4-10. Number of eggs laid by ACP feeding on flush of 3 treatments after 4 days.

CHAPTER 5 GENERAL CONCLUSIONS

The main goal of this thesis was to explore a sustainable pest management method to reduce the spread of citrus greening disease (HLB). The strategy focuses on control of the vector, the Asian citrus psyllid (ACP) that transmits the bacterium associated with HLB. Gene targets (TG) in ACP were selected and fed to the insect in the form of dsRNA to induce an RNA interference (RNAi) response in the cytoplasm of the ACP cell. Complementary sequences to the TG1 will be downregulated, therefore silencing their own essential genes. Double-stranded RNA was created by a potentially deployable viral expression vector, a phloem-limited plant virus that naturally occurs in citrus, called citrus tristeza virus (CTV). The novel attribute of CTV is that it naturally produces fragments of dsRNA in the form of replicative intermediates in the phloem.

The treatment plants used in this project were infected with CTV with either of two different gene inserts, a sequence with a ACP gene target (TG1) or green fluorescent protein (GFP). Genes can be detected up to eight years after inoculation with CTV with the inserted gene fragment (Siddrame Gowda, personal communication). This suggests that CTV can, continuously replicate foreign fragments within its genome for extended periods of time, while still maintaining its virology in the plant.

TG1 can be detected in varying amounts among plants and plant. Younger flush of CTV/TG1-inoculated plants are able to produce higher copy numbers of the TG1 sequence (stage 1 leaves producing an average copy number of 113922159 and stage 2 having an average copy number of 178396778). This is particularly interesting because ACP depend on younger flush for nymphal development and for ovipositional

activity. Potentially, this could mean toxicity would be higher in the stages of the plant which are most vulnerable to feeding and invasion by ACP.

Fragments made by the CTV are both single-stranded and double-stranded fragments, but an RNAi response can only be induced by ACP cells when the insect feeds on the dsRNA fragments created by the CTV containing the ACP TG1 sequence. The fact that the CTV makes the TG1 in the form of dsRNA means that an RNAi response can be induced by feeding to ACP.

Adult ACP mortality is increased when they feed on stage 4 mature leaves of CTV/TG1 plants compared to leaves without the TG1 sequence. Mortality varies among leaves of the same treatment, indicating that CTV/ TG1 sequence quantity is unevenly distributed throughout leaves of the same plant. When the data from one feeding trail were analyzed, ACP mortality appeared correlated with the amount of copy number of the TG1 sequence in the leaf. Due to the fact that the rest of the data do not support this hypothesis (e.g. the leaf with the second highest copy number of the TG1 sequence did not provoke the second highest mortality rate), I conclude that there might be a certain copy number of the TG1 sequence present to induce a high mortality. It would be interesting to test this theory in future experiments by detecting copy number from more leaves that caused high numbers of ACP mortality.

Egg laying ability is apparently not affected when ACP feed on flush containing CTV/TG1. CTV/GFP induced the lowest number of eggs laid which could mean it could be responsible for a non-target effect of ovipositional activity in ACP. Further experiments to analyze if low ovipositional activity is affected by ACP feeding on CTV

infected plants with no inserted gene. To assess if ACP feeding on dsRNA fragments alone is enough alone to cause ovipositional activity change.

RNA interference has been successful in many applications but that novel aspect of using a virus to naturally produce dsRNA fragments that can easily be taken up by ACP when feeding on the phloem of citrus creates a more accessible way to encourage ACP to acquire the dsRNA fragments. The fact that genes such as TG1 can be successfully inserted into the CTV genome and replicated in the phloem of citrus by CTV replication means it could be added and allowed to infect already established groves. It has been noted that different strains of CTV can co-exist inside the same citrus plant (Satyanarayana et al., 2001), which means it will still be able to replicate and produce these dsRNA fragments in established citrus groves, even those already infected with a different strain of CTV. CTV can be infectious for up to eight years in the same plant (Siddrame Gowda, personal communication), which means it can be long-lasting and sustainable in citrus groves. It would be first important to explore how much of the TG1 sequence is necessary to cause a developmental response or induce mortality in ACP feeding on these plants. Choice and no-choice experiments would benefit further investigation of this project because it is possible ACP would avoid plants containing the CTV, so it would not particularly have to induce extremely high mortality to reduce transmission rates of the HLB pathogen.

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BIOGRAPHICAL SKETCH

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