Investigating Vector-Virus-Plant interactions influencing transmission efficiency of *Tomato yellow leaf curl virus* and *Tomato mottle virus* by *Bemisia tabaci*

by

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Abstract

The whitefly *Bemisia tabaci* (Hemiptera: Aleyrodidae) causes crop damage globally by feeding on phloem sap with their piercing-sucking mouthparts, excreting honeydew which promotes growth of sooty mold, and by transmitting viral plant pathogens. Begomoviruses are the largest group within family Geminiviridae and are transmitted by *B. tabaci* in a persistent and circulative manner. *Tomato yellow leaf curl virus* (TYLCV) and *Tomato mottle virus* (ToMoV), are two economically important begomoviruses in the U.S. The overall goals of these studies were to generate new knowledge on vector-virus-plant interactions underlying infection and spread of begomoviruses. The specific objective of the first study presented in Chapter Two is to conduct serial transmission experiments to examine evolutionary relationships of virus-vector and virus-plant interactions responsible for plant infection and whitefly transmission of TYLCV and ToMoV. During each transmission cycle, symptoms were recorded weekly for four weeks, transmission efficiency of whiteflies characterized, virus infections confirmed with PCR, and qPCR was used to quantify titers in the plants. The objective of the experiments in Chapter Three were to further characterize changes in virus titers within individual plants and at different times after inoculation to assess the influence of titer on symptoms and potential for influencing transmission outcomes. Results from experiments in Chapter Two show decreases in viral fitness through time in this closed system, and do not provide significant evidence of adaptation of the virus to the plant or vector after serial transmission. They do, however, provide new information on virus titers of ToMoV, and relationships among virus titers, transmission efficiency, and

symptom expression for these two viruses. Findings of Chapter Three provide new information about titers of TYLCV and ToMoV in different leaf positions of the plant, and at four time points after inoculation. Results from these studies are important to better understand virus-vector-plant interactions, and provide guidelines for conducting experimental evolution studies with begomoviruses and *B. tabaci*.

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Table of Contents

List of Tables

List of Figures

List of Abbreviations

TYLCV	Tomato Yellow Leaf Curl Virus
ToMoV	Tomato Mottle Virus
WF	Whitefly
MEAM1	Middle East-Asia Minor 1
Days post inoculation	dpi
Acquisition access period	AAP
Inoculation access period	IAP
Midgut protein	MGP
Primary salivary gland	PSGs
Coat protein	CP
Replication-associated protein	Rep
Replication enhancer protein	REn
Transcriptional activator protein	TrAP
Nuclear shuttle protein	NSP
Movement protein	MP
Transcriptional gene silencing	PTGS
%	Percent
°C	Degrees in Celsius
s	second

min	Minute
h	hour
g	Gram
mm	Millimeter
cm	Centimeter
m	Meter
ml	Milliliter
µl	Microliter
pH	Potential of hydrogen
×g	Times gravity
LB	Lysogeny broth
DNA	Deoxyribonucleic acid
PCR	Polymerase chain reaction
qPCR	Quantitative polymerase chain reaction
RCA	Rolling circle amplification
Ct	Threshold cycle
ANOVA	Analysis of variance

Chapter One

Literature Review

Whitefly (*Bemisia tabaci*)

Economic importance

Bemisia tabaci Gennadius (Hemiptera: Aleyrodidae) is a cryptic species of whiteflies responsible for severe crop losses worldwide, including tomatoes, pepper, eggplant, cucumber, cotton, tobacco, cassava, beans, okra, watermelon, melon, and ornamentals (Cock 1986a, Oliveira et al. 2001). In the USA, it was estimated that $100-500 million dollars of economic loss were caused by *B. tabaci* in each year from 1991-1995 (Oliveira et al. 2001). These whiteflies directly damage plants by feeding on phloem sap with their piercing-sucking mouthparts, and indirectly because honeydew they excreted onto the surfaces of leaves promotes growth of the sooty mold fungi that can decrease photosynthesis and quality of plants (Byrne 1991, Navas-Castillo et al. 2011). The most severe crop losses result from indirect damage caused by transmission of a large number of economically important viral plant pathogens, including geminiviruses, closteroviruses, carlaviruses and potyviruses (Brown and Czosnek 2002).

Whitefly biology

Whiteflies exhibit incomplete metamorphosis (hemimetabolous development) and complete the following developmental stages in their lifetime: eggs, four nymphal instars and adults (Chapman 1998). Eggs are laid by adults on the underside of leaves. First instars are translucent, flattened and elliptical in shape. After eclosion they crawl to locate a feeding site along a leaf vein where they will attach to the leaf, feed on phloem contents, and remain in place until they complete development to adult (Byrne 1991). The second and third instars are similar in appearance to each other, remain opaque-white, but are different sizes (Gill 1990). Fourth instars are distinct from other instars because they have two red eyes and yellow body pigment are formed (Byrne 1991). The wings start to form at the end of the third instar and exist in the fourth instar (Borror et al. 1989, Henryk and Murad 2011). Adults' wings are white and covered with dust or waxy powder, and the length of forewings and hind wings are almost same (Borror et al. 1989). Adult whiteflies are small with an average length of 1-3mm. It is reported that the average length of females is longer than males (Byrne 1991). Thus, female and male can be visually identified through size.

Growth and development of *B. tabaci* varies with geography, season, temperature and host plants. Under natural conditions in Egypt, an average of 252 eggs and 204 eggs were laid on cotton per female when the daily maximum temperature (DMT) was 28.5℃ and 22.7℃ respectively, but only around 61 eggs were oviposited per female under 14.3℃ DMT (Azab et al. 1971, Byrne 1991). In Arizona, a research showed average 81 eggs were laid on cotton per female of *B. tabaci* at 26.7℃ and 72 eggs at 32.2℃ in incubator with constant light and temprature. Among those eggs, 68% and 75% hatched at 26.7℃ and 32.2℃ respectively. The duration from egg to adult varied from 61.5 days to 16.6 days at 14.9℃ and 30.0℃ respectively, and *B. tabaci* adults live approximately 8 days at 26.7℃ and 11 days and 32.2℃ (Butler et al.

1983). When *B. tabaci* were reared under same constant temperature, development time from egg to adult was 30-40% reduced on sweet potato, lettuce, cucumber and eggplant compare to broccoli or carrot (Coudriet et al. 1985). The rainy season was negatively correlated with development duration of *B. tabaci* on cassava in southern Ivory Coast (Fishpool et al. 1995). Moreover, virus infection status can also have influence on fecundity and development duration of *B. tabaci*. In one study, approximately 15 days were need for individual *Tomato yellow leaf curl virus* (TYLCV) infected *B. tabaci* to develop from eggs to adults while 16 days were need for healthy individuals (Maluta et al. 2014). Another study showed that biotype B of *B. tabaci* that had acquired *Tomato mottle virus* (ToMoV) laid an average of 15 eggs per female while 9 eggs were laid per nonviruliferous female (McKenzie 2002).

Whitefly systematics

B. tabaci is a species complex for which there is evidence that 36 species exist based on sequence variation of proteins and DNA makers (De Barro et al. 2011). Before the availability of molecular techniques, the biotype concept was used to distinguish morphologically indistinguishable populations of *B. tabaci* that exhibited differences in biological characters, including host ranges (Burban et al. 1992), vector competence to transmit plant viruses (Bird and Maramorosch 1978), phytotoxic disorders in certain plant species, interbreeding (Bedford et al. 1994), invasiveness, and insecticide resistance (Brown, Frohlich, et al. 1995).

Among members of the *B. tabaci* species complex, Middle East-Asia Minor 1 (MEAM1 subspecies) previously known as biotype B, is one of the most invasive and destructive biotypes (Ghanim 2014). Biotype B originally comes from the Middle East-Asia Minor region (De Barro et al. 2011) and was first reported as a greenhouse pest on vegetables in Turkey (Cock 1986b,

Broadbent et al. 1989). It caused the first huge global invasion event that spread *B. tabaci* to at least 54 countries distributed across North America, South America, Africa, Oceania, Asia and Europe through ornamental crops (De Barro et al. 2011). The introduction of the B biotype into USA was first reported on poinsettia in Florida greenhouses in 1986 and on field tomatoes in 1987 (Price et al. 1987, Schuster and Price 1987, Broadbent et al. 1989). Since then it has outcompeted and displaced Biotype A (New World), which was widespread before the introduction of Biotype B. More recently, Biotype Q (Mediterranean subspecies) another invasive and destructive biotype, was detected on poinsettias in Arizona in 2004 (Dennehy et al. 2005), and is now found in 25 states across USA, including Florida (Mckenzie et al. 2009).

Whitefly-Transmitted Viruses

Geminiviridae

Geminiviruses cause huge crop and economic loss every year worldwide, and in the Americas, especially Mexico, Central and South America (Polston and Anderson 1995), and comprise the most significant group of pathogens transmitted by *B. tabaci* (Henryk and Murad 2011). There are a total of nine genera in the family Geminiviridae (ICTV 2017), including three well-known genera – *Curtovirus*, *Mastrevirus* and *Begomovirus* (Rojas et al. 2005, Hanley-Bowdoin et al. 2013), established in 1978 (Goodman 1981). Among them, begomoviruses are the largest group within family Geminiviridae (Varsani et al. 2014, Rosario et al. 2015) with at least 322 identified species (ICTV 2017). The structure of all geminivirus viral particles is circular, single-stranded DNA (ssDNA) genome packaged into a virus particle (Zhang et al. 2001). Most genomes of begomoviruses consist of two (bipartite) DNA components, including

Tomato mottle virus (ToMoV), while a only have one (monopartite) DNA component, including *Tomato yellow leaf curl virus* (TYLCV) (Hitefly et al. 1999).

Genome of begomoviruses

The genome, encoding 5-8 proteins, of begomoviruses have been well characterized (Hanley-Bowdoin et al. 2013). The DNA-A and B components of bipartite begomovirses and DNA of monopartite begomovirses are homologous. The genome of A component of bipartite viruses and monopartite viruses encode coat protein (CP), replication-associated protein (Rep), transcriptional activator protein (TrAP), replication enhancer protein (REn), and virulence factor (C4 or AC4). And the genome of B component of bipartite begomoviruses encode nuclear shuttle protein (NSP) and movement protein (MP) (Gutierrez 1999, Hitefly et al. 1999, Rosario et al. 2015). CP controls viral capsid formation and vector transmission (Briddon et al. 1990, Hanley-Bowdoin et al. 2013), and in monopartite viruses also functions as NSP, which is located on DNA B of bipartite viruses (Poornima Priyadarshini et al. 2011). Rep is known to interact with REn (Settlage et al. 2005) for viral replication (Taylor et al. 1999). Repression of Rep leads activation of TrAP expression which is involved in the activation of CP and NSP expression (Hanley-Bowdoin et al. 2013). C4 or AC4 plays an important role in symptom development, induces cell proliferation (Latham et al. 1997), and counteracts post – transcriptional gene silencing (PTGS) (Carvalho et al. 2008, Hanley-Bowdoin et al. 2013). V2 and AV2 proteins also encode a suppressor of PTGS for overcoming the plant defense system (Glick, Zrachya, et al. 2009, Zhang et al. 2012, Hanley-Bowdoin et al. 2013). There is research showing that V2 is involved with cell to cell movement of viral DNA (Poornima Priyadarshini et al. 2011) (Figure 1).

Tomato yellow leaf curl virus (TYLCV)

TYLCV, one of the most destructive tomato-infecting begomoviruses, is a complex of virus species that originated in in the Middle East - Mediterranean region (Cohen and Harpaz 1964, Pan et al. 2012), and was first reported in Israel in late 1930s (Picó et al. 1996). Currently, there are at least 13 main species of TYLCV, mainly including TYLCV-Th, TYLCV-Ch, TYLCV-Sar, TYLCV-Tz, TYLCV-Ng, TYLCV-SSA and TYLCV-Is (Moriones and Navas-Castillo 2000, ICTV 2017), presenting in more than 40 countries from Asia, Australia, Africa, Europe and the Americas (Glick, Levy, et al. 2009). Most TYLCV species differ in their geographic distributions, such as TYLCV-Ch and TYLCV-Th that are only found in China and Thailand, respectively (Moriones and Navas-Castillo 2000). However, some distinct TYLCV can also exist in same area at same times. During 1996-1998, TYLCV-Sr and TYLCV-Is were both found to infect tomato plants in southern Spain at the same time (Sánchez-Campos et al. 1999). In USA, TYLCV-Is was first detected in south Florida in 1997 (Polston et al. 1999), and spread to north Florida and south Georgia rapidly in 1998 (Momol et al. 1999).

TYLCV has very wide host range with more than 30 plant species in over 12 families, including crops, like tomato, tobacco, potato, eggplant, pepper (Picó et al. 1996), bean (Navas-Castillo et al. 1999), ornamentals, and many wild plants and weeds. TYLCV may cause shoots to become distorted with yellowing, leaflets being reduced in size and curled upwards, and plants stunted (Lapidot and Friedmann 2002, Ammara et al. 2017). In the field 20 - 100% yield loss of tomato plants has been reported (Pan et al. 2012). Asymptomatic infections also occur in some crops, and there is research showing that pepper could be infected without showing any symptom on plants and fruits (Polston 2003).

TYLCV is a monopartite begomovirus comprised of 2.7-2.8kb nucleotides (Navot et al. 1991). However, the genome of TYLCV-Th has been characterized as bipartite with A and B components formed by 2751 nucleotides and 2737 nucleotides respectively (Czosnek et al. n.d., Attathom et al. 1994). It is well known that TYLCV is transmitted exclusively in nature by *B. tabaci*. There are also reports showing that TYLCV can be transmitted during mating process between different sexes but same biotype of *B. tabaci* (Ghanim et al. 2007), and from viruliferous whiteflies to offspring (Ghanim et al. 1998). In Ghanim's experiment, TYLCV can be detected in eggs, crawlers and adults from offspring of TYLCV infected whiteflies using PCR (Ghanim et al. 1998). However, the other report showed that gene of TYLCV in offspring can only be found in eggs and nymphs, not in pupa or adults (Pan et al. 2012). In the laboratory, TYLCV can be transmitted artificially via grafting, DNA-coated particle bombardment or agroinoculation using *Agrobacterium tumefaciens* (Scholthof et al. 2011). There is only report on seed transmission of TYLCV-Is in tomato (Kil et al. 2016).

Tomato mottle virus **(ToMoV)**

ToMoV is believed to originate from Florida, USA (Polston and Anderson 1995) and was first observed on tomato crops in Naples, Florida in 1989 (Polston 1993). In 1994, a report showed that it has spread to South Carolina, Tennessee, Virginia (Polston 1995), and Puerto Rico (Brown, Bird, et al. 1995). Compared to TYLCV, ToMoV has fewer host plants, and is only reported to infect tomato, common bean, and tobacco by artificial inoculation (Polston 1993). ToMoV can cause molting, cupping and curling of leaf with yellow mosaic distortion and plant stunting (Polston 1993). Symptoms of ToMoV infected plants are milder than those of TYLCV infected plants, but it has been reported to cause up to 50% crop loss in field (Abouzid et al.

1992). In 1996, ToMoV was found in all tomato-producing areas of Florida, was found to infect up to 95% of plants in a field, and lead to an estimated $125 million dollars of economic loss (EPPO Global Database 2018). ToMoV is a bipartite begomovirus whose genome includes A component constituted by 2601 nucleotides and B component comprised by 2541 nucleotides (Abouzid et al. 1992). ToMoV can be transmitted by biotype B of *B. tabaci* (Schuster et al. 1989, Polston 1993). In the laboratory, ToMoV can be transmitted artificially via agroinoculation using *Agrobacterium tumefaciens* (Rajabu et al. 2018), but there have been no reports of seed transmission in ToMoV (Polston 1993). To date, much fewer studies have been conducted on ToMoV than TYLCV.

Tomato 'Florida lanai' (*Solanum lycopersicum*)

Tomato originated from the Andean region in South America (Bai and Lindhout 2007) and was taken to Europe in the 15th century (Sims 1980). Currently, numerous varieties of tomato are grown worldwide. Tomato is a host of *B. tabaci* and more than 60 distinct begomoviruses (EFSA Panel on Plant Health 2013). The variety, 'Florida Lanai', is one dwarf tomato variety developed by the University of Florida (Augustine et al. 1981), that reaches a height of 30-45 cm with regular leaf and determinate growing habit. 'Florida Lanai' has been recommended and used as a host plant in studies related to *B. tabaci* and viruses, including TYLCV and ToMoV (McKenzie 2002, Momotaz et al. 2007, Nava et al. 2013, Rajabu et al. 2018).

Virus transmission by insects

Viruses need to be moved from one host plant to another to persist in the environment, and insects are the main vectors of plant viruses (Whitfield et al. 2015). There are four basic modes

of transmission by insects: non-persistent, semi-persistent, persistent and circulative, as well as persistent and propagative (Ng and Falk 2006). *B. tabaci* can transmit several different viruses with different modes. It has been reported that viruses from the genuses *Crinivirus* and *Ipomovirus* can be transmitted by *B. tabaci* in a noncirculative and semipersistent manner (Andret-Link and Fuchs 2005) while *Carlavirus* can be transmitted in either semi-persistent or non-persistent manner (Polston et al. 2014).

Begomovirus **transmission by** ***Bemisia tabaci***

Begomoviruses are transmitted by *B. tabaci* in a persistent and circulative manner (Rosen et al. 2015). For bipartite begomoviruses, both DNA-A and DNA-B components, that are packaged into separate virus particles, and must be transmitted together into a single cell in order to initiate replication and subsequent movement out of the cell (Liu et al. 1997). Begomoviruses are phloem-limited and are ingested and subsequently transmitted by *B. tabaci* during feeding events. Once ingested the virus passes through the stylets, esophagus, filter chamber, and enter the midgut. These viruses then cross the membrane of the midgut and circulate through hemolymph to the salivary glands, sometimes aided by endosymbiont derived proteins. After entering the primary salivary gland they must then localize in the salivary ducts where they can enter saliva and be injected into host plants during feeding (Czosnek et al. 2002, Rosen et al. 2015). The minimum acquisition access period (AAP) of TYLCV varied from 15 to 60 minutes while the minimum inoculation access period (IAP) varied from 15 to 30 minutes (Henryk and Murad 2011). It has been reported that TYLCV can be detected in head of biotype B of *B. tabaci* after 10-min AAP, in midgut after 40 min AAP, in hemolymph after 90 min AAP, and in salivary glands after 7h AAP (Ghanim et al. 2001). TYLCV transmission rate of *B. tabaci* to

healthy tomato plants ranged from 0/25 and 13/25 plants infected with 8h and 40h latent period, respectively, and the minimum latent period was 22h (Mansour and Al-Musa 1992). In another study TYLCV transmission by *B. tabaci* to healthy tomato plants increased from 0% to 100% with increasing latent period from 4h to 94h. however, the minimum latent period is only 9h (Ghanim et al. 2001). Generally, there are no increases in transmission efficiency observed after and AAP of 48 hours. Also, when *B. tabaci* was given 24h AAP and 24h IAP to transmit TYLCV to healthy tomato plants, plant infection rate increased from 60% to 100% by increasing the number of whiteflies from 3 to 20 in transmission assays (Mansour and Al-Musa 1992).

Begomovirus transmission by *B. tabaci* is influenced by vector, virus, plant interactions. Different species of *B. tabaci* have been shown to exhibit different transmission efficiencies of begomoviruses. One study showed Biotype Q of *B. tabaci* are more competent vectors than Biotype B due to the ability of Biotype Q to acquire more viral DNA and attain the maximum viral load faster than Biotype B of *B. tabaci* (Pan et al. 2012). Research in Spain showed no difference in transmission efficiency of TYLCV-Sar between biotype B and biotype Q from infected tomato plants to healthy weed plants, or from TYLCV-Sar infected *Datura stramonium* L. (jimsonweed) to tomato plants. However, biotype Q of *B.tabaci* was observed to transmit more efficiently than biotype B from *Solanum nigrum* L. (black nightshade) to tomato plants (Jiang et al. 2004). Different populations of *B. tabaci* have also been shown to exhibit different vector competency to transmit TYLCV (Kollenberg et al. 2014). It is reported that TYLCV-Is is more efficiently vectored by local biotypes of *B. tabaci* than TYLCV-Sr, which caused displacement of TYLCV-Sr by TYLCV- Is as the causative agent of epidemics in tomato plants in Spain (Navas-Castillo et al. 1999).

Gender, age and number of *B. tabaci* can also influence virus transmission efficiency. Research in 2015 showed that females of biotype B and Q of *B. tabaci* carry a higher viral load of TYLCV than males, and that females are more competent vectors (Ning et al. 2015). The ability of one female of biotype B (7 days after emerging to adult) to transmit TYLCV-Is to tomato plants is equal to the ability of five males of the same biotype and age (Ghanim et al. 2001). Plant infection rate of TYLCV-Is by biotype B decreased from 100% to 20% during their adult lifetime (0 to 35 days after emerging to adult) (Rubinstein and Czosnek 1997). TAnother study showed that only 20% of 6-week-old female *B. tabaci* infested healthy tomato plants while 60% of 3 week-old female infested healthy tomato plants. In the same experiment, 3 week-old male *B. tabaci* were not able to infest healthy tomato plants whereas 60% of 3 week-old female infested healthy tomato plants (Czosnek et al. 2001, Henryk and Murad 2011). Plant variety has also been shown to influence acquisition and transmission of plant viruses by *B. tabaci*. Previous research on TYLCV transmission by *B. tabaci* on different tomato varieties showed that transmission was lower in highly resistant tomato plants (Lapidot et al. 2001).
Moreover, viruliferous *B. tabaci* can enhance TYLCV inoculation efficiency by alerting their settling, probing and feeding behavior (Moreno-Delafuente et al. 2013).

Virus quantification

Methods have been developed that allow for the quantification of virus copy number in plant and insect samples. Currently, there are three main categories of methods used to quantify viruses: direct counting of physical viral particles, measuring viral infectivity, and measuring viral protein as well as nucleic acids (Pankaj 2013). Quantitative polymerase chain reaction (qPCR) is a method of measuring viral nucleic acid, offers greater sensitivity than conventional

PCR (Ammara et al. 2017), and has been used to quantify viruses in *B. tabaci* and host plants. Using qPCR assay, Pan et al. found that biotype Q of *B. tabaci* can acquire more TYLCV DNA than biotype B, and biotype Q can carry higher viral loads than biotype B (Pan et al. 2012). In 2015, a study in China showed biotype Q females can acquire significantly more TYLCV than biotype Q males and both sexes of biotype B (Ning et al. 2015). Ning et al. (2015) used qPCR to quantify TYLCV in tomato leaves exposed to one viruliferous *B. tabaci*, and showed that females of biotype B and Q transmitted significantly more virus than males, and that Biotype Q females transmitted a significantly higher amount than all others. This method has also been used to examine titers in leaf tissue. One study showed there is no significant difference in the quantity of TYLCV-Sar among tomato leaves at different positions: 1st, 2nd, 3rd and 4th leaf from the growing point (Mason et al. 2008). It has also been shown that titers of Mld and IL strains of TYLCV in tomato plants peak 20d after agro-inoculation while *Potato yellow mosaic virus* peak 10d after (Péréfarres et al. 2011). Higher titers have also been shown to occur in plants with more severe begomovirus symptoms, including the viruses *Tomato yellow leaf curl virus* – Oman (Ammara et al. 2017), *African cassava mosaic virus,* and *East African cassava mosaic virus* (Naseem and Winter 2016, Kuria et al. 2017).

Vector-virus-plant selection and coevolution

In the real world, viruses are continuously evolving to adapt to new environments, host plants and vectors by generating new genetic variants with altered biological properties. Viral genetic diversity can change quickly due to their high mutation and recombination rates, and reassortment among closely related species. Virus populations frequently endure bottleneck events during with-host progression and host-to-host transmission (Pfeiffer and Kirkegaard 2006,

McCrone and Lauring 2018). Several vector-virus-plant interactions have been hypothesized to impose selection on viral populations during infection, disease progression, vector feeding, acquisition and transmission (McGRATH and HARRISON 1995, Liu et al. 1997, Gray et al. 2014). CP plays an important role in selection, especially imposed by vectors. In *B. tabaci*, CP of *Tomato leaf curl New Delhi virus* and *Cotton leaf curl Rajasthan virus* (CLCuV) interact with midgut protein (MGP) that allow specific entry of virus into hemolymph, and anti-MGP antibody can lead to 70% reduction in ToLCV transmission (Rana et al. 2016). CP can also interact with the GroEL protein of endosymbionts in *B. tabaci*, that provides protects to the virus in the hemolymph (Gottlieb et al. 2010, Péréfarres et al. 2012, Rana et al. 2012). CP of CLCuV can interact with a GroEL protein produced by the endosymbiont *Arsenophonus* in *B. tabaci* (Rana et al. 2012). The GroEL protein of *Hamiltonella*, has only been reported in biotype B of *B. tabaci*, and has been shown to interact with CP of TYLCV and is involved in transmission. The Q biotype scarcely transmits TYLCV, and it is hypothesized that this is due to lack of *Hamiltonella* in biotype Q (Gottlieb et al. 2010). CP of TYLCV can interact with primary salivary gland (PSGs) of *B. tabaci;* accumulation of the virus in PSGs is required for successful viral transmission (Wei et al. 2014). Not only vectors, but plants can also impose selection on viruses. In host plants, gene silencing is a major defense mechanism of prevention of viral gene expression by degrading viral RNA (Ratcliff et al. 1999). C4 or AC4 in begomoviruses is correlated with overcoming post – transcriptional gene silencing (PTGS) (Carvalho et al. 2008, Hanley-Bowdoin et al. 2013), symptom development, and induces cell proliferation (Latham et al. 1997). V2 and AV2 proteins can also encode a suppressor of PTGS for counteracting plants defense (Glick, Zrachya, et al. 2009, Zhang et al. 2012, Hanley-Bowdoin et al. 2013).

Conclusion

Begomoviruses, including TYLCV and ToMoV, transmitted by *B. tabaci* cause significant economic losses in many crops worldwide. Although TYLCV is the focus of many research articles, no one has conducted experiments to examine coevolution of *B. tabaci*-*Begomovirus*-plant interactions. There is also no information about virus titers in ToMoV infected plants, or the quantities of both DNA-A and DNA-B components in plants infected with bipartite begomoviruses. The first objective of this study is to conduct experimental evolution studies to examine the relationships of virus-vector and virus-plant interactions including transmission efficiency, symptom expression and virus titers of TYLCV and ToMoV after repeated transmission with *B. tabaci* in tomato. The second objective of this study is to examine the relationship between virus titer of plant tissue and symptoms of leaves at different location on the plant, and at different time point after TYLCV and ToMoV inoculation.

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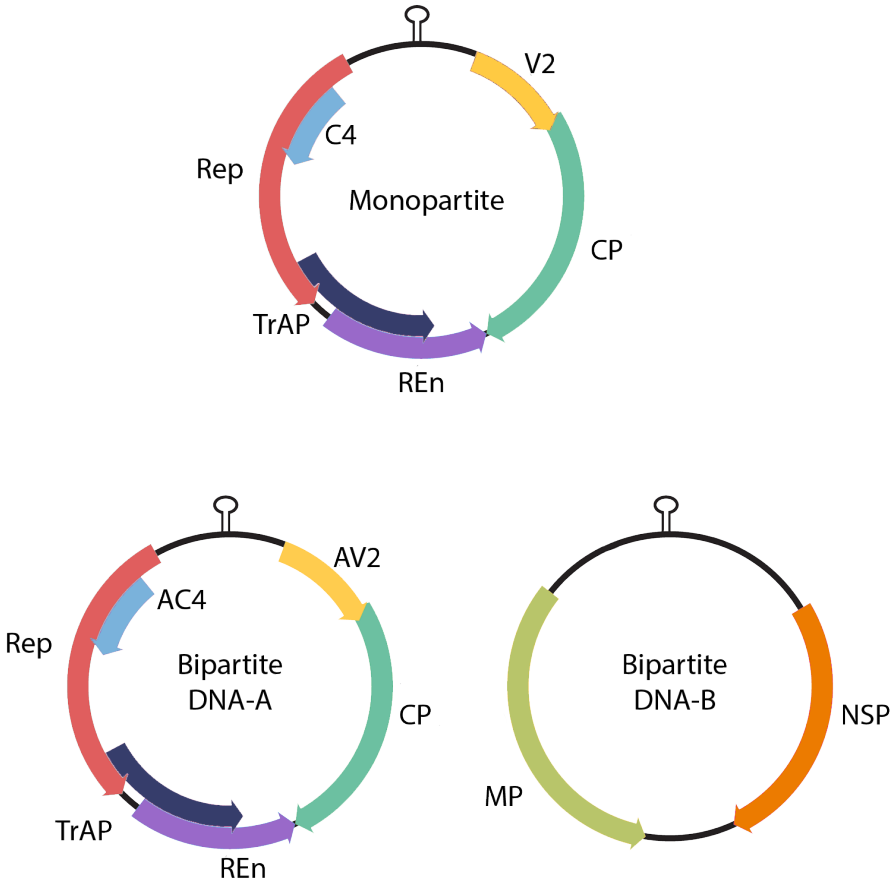

Fig. 1. Genome organization of *Begomoviruses* including monopartite and bipartite. Arrows encode capsid (CP), replication-associated (Rep), transcriptional activator (TrAP), replication enhancer (REn), virulence factor (C4 or AC4), V2 protein, AV2 protein, nuclear shuttle (NSP) and movement protein (MP) proteins.

Chapter Two

Evaluation of virus transmission efficiency, symptom development and virus titer of *Tomato yellow leaf curl virus* and *Tomato mottle virus* during serial transmissions by *Bemisia tabaci*

Introduction

Whitefly-transmitted begomoviruses, including *Tomato yellow leaf curl virus* (TYLCV) and *Tomato mottle virus* (ToMoV), are the largest group within the family Geminiviridae (Varsani et al. 2014, Rosario et al. 2015), with at least 322 species (ICTV 2017). A large number of viruses in this genus cause severe economic damage and threaten crop production worldwide, especially in the tropics and subtropics (Navas-Castillo et al. 2011). Begomoviruses are transmitted in nature by the whitefly, *Bemisia tabaci* Gennadius (Hemiptera: Aleyrodidae), which is a cosmopolitan and genetically diverse complex of at least 36 morphologically indistinguishable species (De Barro et al. 2011), of which several are considered to be among the world's 100 worst invasive species (Lowe et al. 2004). Globalization and world trade have resulted in the spread of multiple invasive *B. tabaci* species into new areas around the world, often concomitant with spread of the begomoviruses they transmit. These biological invasions change ecological relationships between endemic viruses, vectors and host plants, and are often associated with new and emerging viral diseases, including many *Begomovirus* species (Fargette et al. 2006,

Péréfarres et al. 2012, Fereres 2015). High mutation rates, recombination and reassortment are characteristics of begomoviruses reported to promote adaptive genetic variation that can be selected for new and advantageous traits that enable them to acquire new hosts, vectors, and move into new environments (Elena et al. 2011, Péréfarres et al. 2012, Bedhomme et al. 2015). Introduction of new *B. tabaci* species into an area may promote spread and evolution of viral populations if the introduced vector species is a more competent vector, has a larger population size, different dispersal behavior, or has a larger host plant range than endemic species (Fereres 2015). A better understanding of factors driving the evolution of vector-virus-plant interactions are needed to address the emergence of viral pathogens threatening crop production worldwide.

Begomoviruses are transmitted by *B. tabaci* in a persistent and circulative manner (Czosnek et al. n.d., Ghanim 2014, Rosen et al. 2015). A series of vector-virus interactions are required for the virus to circulate through and be transmitted by the whitefly vector. Whiteflies acquire begomoviruses when they feed on the phloem of infected plants. Once ingested, virus particles pass through the stylets, esophagus, filter chamber, and enter the midgut of *B. tabaci* where they bind to midgut proteins and cross the membrane into the hemolymph. They then circulate through the hemolymph to the salivary glands, a process that may be assisted by proteins produced by whitefly endosymbionts that bind to virus particles and protect them from degradation until they reach the salivary glands (Czosnek and Ghanim 2012, Péréfarres et al. 2012). Reaching the salivary gland does not ensure transmission; once inside the salivary glands virus particles must localize into the salivary ducts where they can enter saliva and be injected into host plants during subsequent feeding (Czosnek et al. 2002, Rosen et al. 2015). Additionally, one begomovirus has been reported to localize in *B. tabaci* ovaries and be transovarially

transmitted to progeny in some (Ghanim et al. 1998, Bosco et al. 2004, Pakkianathan et al. 2015, Wei et al. 2017), but not all studies (Becker et al. 2015, Sánchez-Campos et al. 2016).

Begomoviruses are single-stranded circular DNA viruses containing one (monopartite) or two (bipartite) genome segments for which a suite of genes have been identified to play a role in plant infection, intra-plant movement, symptom development, and vector-transmission. *Begomovirus* genomes encode 5-8 proteins (Hanley-Bowdoin et al. 2013) (Figure 1). The DNA-A genome segment of bipartite viruses and monopartite viruses encode coat protein (CP), replication-associated protein (Rep) as well as replication enhancer protein (REn), transcriptional activator protein (TrAP), virulence factor (C4/AC4 for mono, and bipartite, respectively), and V2/AV2 protein for mono, and bipartite, respectively. A nuclear shuttle protein (NSP) and movement protein (MP) are encoded by the DNA-B component of bipartite viruses, whereas the CP of monopartite viruses encodes movement functions (Gutierrez 1999, Hitefly et al. 1999, Rosario et al. 2015). Bipartite virus genome segments, DNA-A and a DNA-B, are packaged separately into individual virus particles (Hitefly et al. 1999) (Zhang et al. 2001),which means that both genome segments must be transmitted into a cell for the virus to both replicate and initiate cell-to-cell and intra-plant long distance movement (Liu et al. 1997).

Several vector-virus-plant interactions have been hypothesized to impose selection on viral populations during infection, disease progression, vector feeding, acquisition and transmission (McGRATH and HARRISON 1995, Liu et al. 1997, Gray et al. 2014). Vector–virus and virus-plant interactions may impose selection on CP genes that have been shown to be responsible for virus particle assembly during plant infection (Noris et al. 1998), vector transmission, vector specificity and transmission efficiency of begomovirus*es* (Briddon et al. 1990, Azzam et al. 1994, McGRATH and HARRISON 1995, Liu et al. 1997, Noris et al. 1998, Höhnle et al. 2001).

Although single CP mutations have been shown to eliminate both whitefly transmission and virus particle assembly in plants (Noris et al. 1998), not all mutations affect transmission and plant infection processes similarly (McGRATH and HARRISON 1995), suggesting plants and vectors may select for different traits (Tian et al. 2017). Selection may also result from membrane interactions of virus particles with the insect midgut (Rana et al. 2016), endosymbiont proteins in the hemolymph (Gottlieb et al. 2010, Péréfarres et al. 2012, Rana et al. 2012), localization in salivary glands (Noris et al. 1998, Caciagli et al. 2009, Wei et al. 2014), and entry into ovaries (Wei et al. 2017) during circulation in the vector. Evidence for vector imposed selection on the CP and vector-virus coevolution includes a reported loss of vector transmissibility after serial vegetative propagations of a *Begomovirus* in the absence of a vector (Liu et al. 1997), and increased transmission efficiency of viruses by local vector populations compared to vectors collected from different geographies (McGRATH and HARRISON 1995, Maruthi et al. 2002).

Plant-insect and plant-virus interactions may also impose selection on viral populations during plant infection, and could indirectly influence vector-virus interactions associated with transmission outcomes (Gutiérrez et al. 2012, Eigenbrode et al. 2017, McCrone and Lauring 2018). Adaptations of whiteflies and begomoviruses that subvert plant defense pathways required for host utilization may have direct and indirect effects on tri-partite interactions. Viral proteins C4/AC4 counteracts post–transcriptional gene silencing (PTGS) plant defense mechanisms (Carvalho et al. 2008, Hanley-Bowdoin et al. 2013), and V2/AV2 proteins can act as a suppressor of PTGS (Glick, Zrachya, et al. 2009, Zhang et al. 2012, Hanley-Bowdoin et al. 2013). Several studies have documented increased virulence of begomoviruses that counteract PTSG in plant hosts (Glick, Zrachya, et al. 2009, Zhang et al. 2012, Hanley-Bowdoin et al. 2013,

Li et al. 2014). Whiteflies have also evolved mechanisms to overcome host plant defenses. Changes in whitefly settling behavior, feeding, and increases in whitefly fitness due to altered plant defense responses have been observed, but is variable among studies with different vector species-virus strain- host plant variety combinations (He et al. n.d., McKenzie et al. 2002, Colvin et al. 2006, Jiu et al. 2007, Li et al. 2011, Wang et al. 2012, 2017, Chen et al. 2013, Luan et al. 2014, Moreno-Delafuente et al. 2013, Shi et al. 2013, Fang et al. 2013, Liu et al. 2013, Luan et al. 2013, Su et al. 2015, 2016, Davis et al. 2015, Legarrea et al. 2015, Yan et al. 2016). This research on plant-virus-vector interactions is providing valuable insights into factors and mechanisms responsible for the observed outcomes and provides support for long-standing coevolutionary relationships among these organisms.

Virus-vector-plant-environment interactions influence local adaptation and coevolutionary dynamics underlying epidemiological patterns and processes associated with plant viral disease incidence, spread, severity and emergence, however, few experimental evolution studies have been conducted on different insect vectors of plant pathogens. One recent study on plant virus evolution during serial transmission of *Soybean dwarf virus* by *Aulacorthum pisum* Harris and *Nearctaphis bakeri* Cowen, aphid vectors on Pea (*Pisum sativum* L.) and soybean (*Glycine max* L.), respectively, reported increased symptoms and titers in both plant species over time, suggesting viral adaptation to plants (Tian et al. 2017). Evidence for selection by the vectors was inconsistent. Increased transmission efficiency was only observed for *A. pisum* on pea; transmission by *N. bakeri* on soybean decreased over time and was lost in 6-7 passages. These results provide evidence for vector and plant induced selection on viral populations, and suggest that differences in the strength of selection exist among different host plant and vectors, and between vectors and host plants. The effects of serial transmission on adaptation of viruses to

their plant hosts and vectors have not been examined in *B. tabaci*-*Begomovirus*-plant systems. The objectives of this study were to serially transmit TYLCV and ToMoV with *B. tabaci* and quantify virus titers, transmission efficiency, and symptom severity through time.

Materials and Methods

Bemisia tabaci colony

A *B. tabaci* colony was started using individuals collected from a greenhouse infestation of tomatoes in 2016. This colony was reared on *Solanum melongena* L. (Pinstripe Hybrid eggplants from Park Seed, Greenwood, SC), a non-host of TYLCV and ToMoV to ensure that whiteflies from the colony were free of these viruses. Whiteflies were reared in 1.6m×0.7m×1.6m insect cages covered with 100 micon screen (Econet 1515, AB Ludvig Svensson, Charlotte, NC) in a greenhouse. Separate cages held adults and immature life stages. Plants used for insect rearing were grown in an environmental chamber (Percival Scientific Inc., Perry, IA) with a photoperiod of 24h light at a temperature of 25.0℃ and a relative humidity of 80±5%, in the Plant Science Research Center in Auburn, AL, USA to keep them free of insects and pathogens before use in the greenhouse. Eggplants were sown in six pack trays (The HC Companies Inc., Middlefield, OH) and transplanted at the four-leaf stage to six inch pots (The HC Companies Inc., Middlefield, OH). Healthy plants were placed in the cage housing adults for 3-4 days, then adults were then removed, the infested plants were transferred to a separate cage, and a new set of healthy plants was placed in the adult cage for another 3-4 days. Eggs from plants containing 3-4 day old cohorts of whiteflies emerged as adults 18-24 days later, depending on temperature. Only 1-3 day old female whiteflies were used in this experiment.

The *B. tabaci* species of this colony was identified as MEAM1/Biotype B by sequencing a partial mitochondrial cytochrome oxidase subunit I gene fragment (COI) from 20 individuals, and using the established phylogenetic classification (Simon et al. 1994, Shatters et al. 2009, Boykin et al. 2012, Boykin and De Barro 2014). DNA was extracted from a single whitefly by adding an individual insect to a 1.5ml tube containing 40µl of lysis buffer (0.01M Tris-HCl, pH 8.4; 0.001M EDTA pH, 8.0; 0.3% Triton-X; 0.1mg/ml Proteinase K) and grinding the individual against the side of the tube with a micropipette tip. Samples were incubated at 95^{o}C for 10min in a T100^{TM} Thermal Cycler (Bio-Rad Laboratories, Inc., Hercules, CA), and then a 1:10 dilution was used as a DNA template for PCR using the B-tab universal primers (Table 1). Before sequencing, samples were processed using ExoSAP-IT (ThermoFisher Cat. No. 78200) according to manufacturer's instructions, and bi-directionally sequenced using Sanger sequencing (ETON biosciences).

Host plants of viruses

Solanum lycopersicum L., variety 'Florida Lanai', was used as a host plant for TYLCV and ToMoV (Rajabu et al. 2018). Florida Lanai tomato seeds were sown in six pack trays (The HC Companies Inc., Middlefield, OH) and grown in an environmental chamber (Percival Scientific Inc., Perry, IA) located in the Plant Science Research Center in Auburn, AL, USA. The seeds were grown with a photoperiod of 24h light at a temperature of 25.0℃ and a relative humidity of 80±5%, in an environment free of insects and viruses. These plants were transferred into 11.5 cm pots (The HC Companies Inc., Middlefield, OH) when they reached the two-true leaf stage and were ready for use in the experiment.

Agrobacterium inoculation

TYLCV and ToMoV *Agrobacterium tumefaciens* infectious clones (Reyes et al. 2013) were used to initiate virus infection in tomato plants. There were 3 treatments, which included TYLCV, ToMoV and non-viral control. Four clones were used in this experiment, each contained one genome segment: the TYLCV genome, ToMoV DNA-A genome, ToMoV DNA-B genome, or an empty pMON721 vector to use as a control. The isolate for TYLCV was originally collected from the Dominican Republic and the ToMoV isolate is originally collected from Florida, USA.

The experimental design of *Agrobacterium* inoculation included 3 biological replicates. Each replicate consisted of 10 tomato plants. Tomato plants with four-true leaves were used to initiate infections of TYLCV and ToMoV using the infectious clones. *Agrobacterium* clones of pMON721, TYLCV, ToMoV-A and -B components were grown separately in 10ml of autoclaved LB medium, pH7.5 (Sigma Cat. No. L9234), with 10µl Spectinomycin in 15ml sterile tubes. They were incubated in a Classic C25 incubator shaker (New Brunswick Scientific Co., Inc., Edison, NJ) at 29℃ with constant shaking for 48h until medium saturation (OD=0.9). Cultures of ToMoV-A and -B components were mixed at 1:1 ratio in a 50ml sterile tube before agroinfiltration. Agroinfiltrations were performed by loading *Agrobacterium* clones in 1 ml syringes (BD Luer-Lok^{TM}, Franklin Lakes, NJ) with 0.26×13mm TSK STERiJECT hypodermic needles (TSK Laboratory, Japan). The clones were inoculated into the plant by piercing the area between apical meristem and the first nodes approximately 5 times while slowly expelling 200-250µl of *Agrobacterium* liquid broth with the syringe. Inoculated tomato plants were placed into trays (Jiffy, Norton, MA) with clear domes (Jiffy, Norton, MA) for 48h to increase the relative humidity. All experiments were conducted in incubators (Percival Scientific Inc., Perry, IA) with

a photoperiod of 16:8 light:dark cycle at a temperature of 25.0℃ and a relative humidity of 75±5%. TYLCV, ToMoV, and pMON721-infected plants were held in different incubators.

Virus transmission by *Bemisia tabaci*

After agroinfiltrations, all transmissions of TYLCV and ToMoV were performed using *B. tabaci*. Each of the three bio-replicates initiated during the agroinoculation were maintained and transmitted independent of the other bio-replications. Only one bio-replicate of control plants was maintained during whitefly transmissions due to space constraints, and these ten control plants were held in the incubator with the TYLCV infected plants. Thirty days post inoculation (dpi), three virus infected plants per bio-replicate were selected as source plants for the acquisition access period (AAP) and were put into a 30cm×30cm×30cm cube BugDorm (MegaView Science Co., Ltd., Taiwan). A total of 200-250 non-viruliferous whiteflies from the greenhouse colony were released into the cube cage and allowed an AAP of 48h. Then 100 viruliferous whiteflies were collected from the cube cage and released in a 58.4cm×41.3cm×15.2cm plastic container (Sterilite Corporation, Townsend, MA) modified to allow for air flow and release of whiteflies (Figure 2.1). Each container held 10 healthy two-true leaf tomato plants for a 48h inoculation access period (IAP). Plastic containers were sealed with parafilm (PARAFILM® M, Neenah, WI) to prevent whiteflies escaping and each bio-replicate was kept separately. Whiteflies were disturbed two times per day to encourage dispersal and an even exposure of plants to viruliferous whiteflies. One hundred non-viruliferous whiteflies from the greenhouse were released onto 10 healthy tomato plants as a control for these experiments. spiromesifen (Oberon®, Bayer Crop Science, Research Triangle Park, NC) at a rate of 7 fl oz/A was sprayed on tomato plants using a handheld sprayer (GardenPlus, China) after 48h to kill

whiteflies, and 5-6 days later to kill immatures. All infected plants from the previous round of transmission were held at room temperature 23°C under lights set to a photoperiod of 11:13 light:dark cycle (due to space constraints) in 370 Funchess Hall in Auburn, AL, USA until successful transmission to the next round was confirmed.

Quantifying transmission by individual whiteflies using sucrose sachets

The transmission rate among individual whiteflies during each round of transmission was tested using 20 whiteflies from the AAP of each bio-replicate concurrent with the IAPs. An artificial feeding medium was made by dissolving 15g of granulated sugar (Alfa Aesar, Ward Hill, MA) in 100ml water with 15µl 64pl green food coloring (McCormick®, Hunt Valley, MD) to make a 15% sucrose solution. A volume of 16µl was pipetted onto one layer of stretched parafilm (PARAFILM® M, Neenah, WI) on the lid of a 1.7ml tube, and then another layer of stretched parafilm was placed over sucrose solution to form a membrane (Figure 2.2). Individual whiteflies from the AAP were introduced to the 1.7ml tubes with sucrose sachets on the same day the IAP was initiated. Whiteflies were held for 48h in the same incubators where the IAP plants were housed. After a 48h IAP, whiteflies were removed and sucrose sachets were stored in the -20℃ freezer (VWR) until testing for virus.

Symptom monitoring and scoring

Symptoms of all tomato plants in the transmission experiments were monitored at 7, 14, 21 and 28dpi. Plant height was measured from the base of the plant at the soil line to the apical meristem. Visual assessments of symptoms were conducted using a 1-4 damage scale and each plant was photographed for reference. The symptom severity rating scale for TYLCV-infected

plants was: (1) no visible symptoms, inoculated plants show the same growth and development as non-inoculated plants; (2) very slight yellowing of leaflet margins on apical leaf; (3) moderate leaf yellowing, curling and cupping, with some reduction in size, yet plants continue to develop; (4) severe plant stunting and yellowing, and pronounced cupping and curling; plant growth stops (Figure 2.3). The symptom severity rating scale for ToMoV-infected plants was: (1) no visible symptoms, inoculated plants show the same growth and development as non-inoculated plants; (2) very slight yellowing, minor cupping on apical leaf; (3) slight yellowing, cupping and curling; (4) some yellowing, plant stunting, and pronounced cupping and curling on multiple leaves (Figure 2.3).

Plant tissue sampling and DNA extraction

All tomato plants were sampled at 28dpi so that infection status could be confirmed using PCR. Four leaf discs were taken per plant using a 6mm diameter 577-CC Premium 1-Hole Punch (Staples®, Birmingham, AL). A separate sterilized hole punch was used for each plant. Hole punches were cleaned with 10% bleach after use, and groups of hole punches were only used to sample one virus throughout the course of the experiments. Two leaf discs were taken from the 1st fully expanded leaf, 1 leaf disc was taken from 2nd fully expanded leaf, and 1 leaf disc was taken from 3rd fully expanded leaf (count from top to bottom of tomato plants). Four leaf discs from one tomato plant were placed in a 2ml microcentrifuge tube with a 2.3mm diameter stainless steel ball (BioSpec Products, Inc., Bartlesville, OK). Liquid nitrogen was used to flash freeze plant tissue samples, and samples were stored at -80℃ in a Revco UxF freezer (Thermo Fisher Scientific Inc., Waltham, MA) until DNA extraction.

Frozen plant tissue samples were ground into powder using a Mini-BeadBeater™ (BioSpec Products, Inc., Bartlesville, OK). Samples were placed in liquid nitrogen during transport to and from the bead beater. The DNeasy® Plant Mini Kit (QIAGEN, Hilden, Germany) was used to extract DNA. A master mix including 400µl Buffer AP1 and 4µl RNase A was aliquoted into each sample and incubated in a water bath at 65℃ for 10min to lyse plant tissue samples. The sample was incubated for 5min on ice after 130µl Buffer P3 was added for the neutralization phase. After incubation, the lysate was centrifuged in a Heraeus Pico 17 Centrifuge (Thermo Fisher Scientific Inc., Waltham, MA) for 5min at 17000×g. The liquid was pipetted into the QIAshreedder spin column and centrifuged for 2min at 17000×g to filtrate cell debris. The flow-through (400µl - 450µl) was transferred into new 1.7ml microcentrifuge tube, and 1.5 volumes of Buffer AW1 was added to adjust pH and salt conditions of solution containing plant DNA. DNeasy Mini spin columns were used to bind DNA into the silica membrane by centrifuging mixture for 1min at 6000×g, 2 times. A DNeasy Mini spin column was then placed into a new 2ml collection tube, and 500µl Buffer AW2 was added into the spin column followed by 1min centrifuging at 6000×g to wash the DNA. The flow-through was discarded and another 500µl of Buffer AW2 was then added to the DNeasy Mini spin column one more time and centrifuged for 2min at 17000×g to dry the membrane. The DNeasy Mini spin column was then transferred to a new 1.7ml microcentrifuge tube, and 25µl Buffer AE was added directly onto the spin column membrane, incubated for 5-10 min at room temperature (15 - 25°C), and centrifuged for 1 minute at 6000×g to elute the DNA. The last step was then repeated once again for a final volume of 50µl. DNA samples were stored in -20℃ freezer (VWR). The DNA concentration was quantified using a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA) according to manufacturer's instructions.

Virus detection in sucrose sachets and plant samples

Rolling circle amplification (RCA) followed by PCR was used to detect viral DNA in sucrose sachets that had been fed on by putatively viruliferous whiteflies from the AAPs. RCA was performed using Illustra TempliPhi 100/500 Amplification Kits (GE Healthcare Life Sciences, UK) to amplify virus DNA in sucrose sachets. A 2.5µl of sample buffer was added to 2µl sucrose solution and heated to 95°C for 3min in a T100^{TM} Thermal Cycler (Bio-Rad Laboratories, Inc., Hercules, CA) to gently lyse the sample. A 2.6µl reaction, including 2.5µl of reaction buffer and 0.1µl of enzyme, was then aliquoted into each sample. Samples were then placed in the same thermal cycler where they were incubated at 30°C for 18h, followed by 65°C for 10 min to inactivate the DNA polymerase.

All plant tissue samples and RCA products of sucrose sachets were tested for virus infection using PCR. Amplification of TYLCV-DNA was achieved using primers TYLCV convFor and TYLCV convRev (Rajabu et al. 2018) (Table 2.1). ToMoV-DNA was amplified using primers TomoV-convFor and TomoV-convRev (Rajabu et al. 2018) (Table 2.1) for the ToMoV-A component. Each 20 µl PCR reaction contained: 1µl DNA template (20-100 ng/ µl) from either plant DNA extraction or RCA, 14.9 µl H_2O, 2 µl 10x NH4 buffer, 0.8 µl 50mM $MgCl_2$, 0.4 µl 10µM each of primers, 0.4 µl 10mM deoxynucleotide triphosphates (dNTPs) and 0.1µl Taq DNA polymerase (5u/ µl) in a 0.2ml PCR tube.

PCR reaction conditions included an initial denaturation of template DNA for 5 min followed by 25 cycles of denaturation at 95℃ for 20s, primer annealing at 58℃ for 20s and extension at 72℃ for 20s. The final extension was run for 5 min at 72℃ and the reaction held at 12℃ in a T100^{TM} Thermal Cycler (Bio-Rad Laboratories, Inc., Hercules, CA). To visualize the

amplified product, 2µl of a solution containing 2µl of gel red and 1ml 6x gel loading dye was added to 10µl PCR product. These samples were then run on a 2% agarose gel in 1× TAE buffer and visualized using UV light.

Generating the Standard Curve for qPCR absolute copy number

Plasmids pNSB1906, pNSB1877 and pNSB1736 containing cloned ToMoV-A, ToMoV-B and TYLCV sequences, respectively, were used to generate standard curves for absolute DNA quantification. The DNA concentration of plasmids was measured with a Qubit 3.0 Fluorometer (Thermo Fisher Scientific Inc., Waltham, MA). Copy number per microliter was calculated from the ng/µl concentration using the formula: copy number = [(DNA ng) x (6.0221×10^{23} molecules/mole)] / [(length of dsDNA amplicon x 660g/mole) x 1×10^{9}]. The lengths of our plasmids were 9,830bp, 10,422bp, and 10,146bp for ToMoV-A, ToMoV-B, and TYLCV, respectively. Plasmid DNA was also run on a gel to make sure there was no other DNA than the specific plasmids. Serial 1:10 dilutions of the plasmid DNA were made in which a genomic viral DNA was present at 30000000 copies, 3000000 copies, 300000 copies, 30000 copies, 3000 copies, 300 copies and 30 copies to be included in qPCR experiments. The threshold cycle (Ct) values of three replicates of each standard-dilution and the log of the total DNA in each sample were used to obtain the standard curves by linear regression analysis. A qPCR standard curve was generated to quantify the unknown samples in every 96-well plate, and each 96-well plate was used for only one virus.

Quantitative PCR (qPCR) amplification of viral DNA

The virus titer of AAP plants was quantified using qPCR. The aforementioned primers were used for quantification of TYLCV and ToMoV-A, and a portion of ToMoV-B was quantified using primers ToMoV-B-Fw and ToMoV-B-Rv (Table 2.1). All preparations of qPCR were performed in the laminar flow hood without light due to the photosensitive reaction of the Syber Green, and qPCR runs were performed on C1000 Touch^{TM} thermocycler (Bio-Rad Laboratories, Inc., Hercules, CA) with CFX96^{TM} Real-Time System. DNA templates were made without dilution or made with 1:5 or 1:10 dilution to make sure Ct values matched standard curves, and three replications of each DNA sample were performed. The amplification reactions were performed in a total volume of 20µl containing 4.6µl sterile H_2O, 10µl iTaq^{TM} Universal SYBR® Green Supermix (Bio-Rad Laboratories, Inc., Hercules, CA), 0.2µl of 10µM virus forward primer and 0.2µl of 10µM virus reverse primer, and 5µl template DNA per qPCR reaction.

The amplification program consisted of a 3min initial denaturation at 95°C followed by 40 cycles of 10s at 95°C for denaturation, 10s at respective annealing temperature and 20s at 60°C for elongation. A final melt step was performed for 5s at 95°C and 5s at 60°C followed by heating to 95°C for 5s with continuous fluorescence measurement. The total viral copy number per ng total DNA was calculated by comparing the average Ct value of triplicate reactions of each sample to standard curves.

Statistical analysis

Statistical analyses were performed using PROC GLIMMIX in SAS version 9.4 (SAS Institute, Cary, NC). All responses were analyzed using the normal probability distribution. Analyses of virus titers, proportion of plants infected, and transmission to sucrose sachets were performed using a repeated analysis ANOVA because the same viral population of each bio-

replicate was sampled through time. Symptom severity ratings and plant heights were analyzed using ANOVA because they were either compared at a single time point, or analyzed as the change in symptoms between the first and last observation period. Regression analysis was used to examine possible correlations between virus titer and transmission efficiency, and between ToMoV-A and ToMoV-B titers. A significance value of $P < 0.05$ was used for all analyses.

Results

Serial transmissions of each biological replicate were conducted for each virus. Plants infected using *Agrobacterium* clones are referred to as Round 0, and each subsequent whitefly transmission labeled sequentially according to the number of times virus had been transmitted using whiteflies. A total of three whitefly transmissions were completed for each bio-replicate of ToMoV (Round 1 – Round 3) before we were unable to successfully transmit the virus after two attempts. Four serial transmissions for each bio-replicate of TYLCV (Round 1 – Round 4) were completed. Infection status of all plants was confirmed 28 dpi and AAPs were always performed 30 days after the previous transmission attempt, however, some transmissions had to be repeated due to failed attempts. Therefore, transmission to the next round occurred 30-107 days after the initial inoculation for Round 0 (Table 2.2). There were no significant differences between bio-replications for either virus, therefore, data were pooled for analysis.

Virus titer of tomato plants

All virus standard curves used to quantify absolute copy number of either TYLCV, ToMoV-A or ToMoV-B were linear with a correlation coefficient value of $R > 0.99$ (Figure 2.4). A series of analyses were conducted to examine changes in titers across rounds of transmission,

compare titers of both viruses, and compare titers of ToMoV-A and ToMoV-B in plant samples collected 28 dpi, and from plants that were used in the AAPs 30-107dpi. The results of an ANOVA to analyze virus titers across rounds for TYLCV and ToMoV infected plants, separately, showed there was no significant difference in titers among Rounds 0-3 and Rounds 0-2, for TYLCV or ToMoV, respectively (Table 2.3). A second analysis was conducted to examine the main effect of the interaction between virus and round on observed titers. In this analysis, titers of TYLCV infected plants were numerically higher than ToMoV infected plants in all rounds except round 1, but these differences were not statistically different during any round of transmission (7,50, F=0.92, *P*=0.4969). Although there were no differences in the average titer value, the variation around the means increased from Round 0 to the last round of transmissions, and standard errors of ToMoV were numerically larger than those for TYLCV (Figure 2.5). Next, a regression analysis was conducted to examine the relative amounts of virus titer of ToMoV-A and ToMoV-B. The titers of ToMoV-A and ToMoV-B were significantly positively correlated to each other (N=9, F=11.24, Coeff=58.98, *P*=0.0122, R^2=0.6163).

The next analyses conducted were to compare titers among AAP source plants used in the experiments at the time of transmission 30-107dpi (Table 2.4). In the first analysis, changes in virus titers of AAP plants were examined across rounds for TYLCV, ToMoV-A and ToMoV-B, separately. There were no significant differences in virus titer of TYLCV or ToMoV-B in AAP plants among any round (Table 2.4). The titers of ToMoV-A were significantly different; titer of ToMoV-A in Round 1 was significantly higher than Round 0 and Round 2, but there was no significant differences between Round 0 and Round 2. Standard errors of virus titer increased through rounds, and standard errors of ToMoV were always larger than TYLCV (Figure 2.6).

Generally, virus titers of TYLCV were numerically higher than ToMoV even though there were no statistical differences.

Virus transmission efficiency of whiteflies

Virus transmission efficiency during serial transmission experiments was examined by quantifying the proportion of infected plants from the IAPs, and the proportion of infected sucrose sachets. There was a significant reduction in the number of infected plants and sucrose sachets of TYLCV across rounds (Table 2.4, Figure 2.7). Proportion infected of ToMoV plants also significantly decreased through time. The proportion of plants infected was always higher than the proportion of sucrose sachets containing virus, but this is likely due to only one whitefly feeding on each sucrose sachet, compared to multiple whiteflies feeding on each plant during the IAP. The proportion of sucrose sachets containing TYLCV was significantly different among rounds, were always numerically lower than the proportion of infected plants, and declined over time. The proportion of sucrose sachets testing positive for ToMoV-A was not significantly different among rounds, and was always below 0.10. To further examine this relationship, a regression analysis was conducted to examine the proportion of infected plants and sucrose sachets containing virus. A positively significant correlation existed for TYLCV (N=15, F=5.39, Coeff=42.75, P=0.0372, R^2=0.293), but was not observed between plants and sucrose sachets with ToMoV (N=9, F=0.41, Coeff=87.97, P=0.5432, R^2=0.0551).

Effect of virus titer on virus transmission efficiency of whiteflies

Regression analyses were conducted on titers of each virus separately to examine: 1) the relationship between titers of AAP plants and the proportion of plants infected during the

corresponding IAP; 2) the titers of AAP plants and the proportion of sucrose sachets containing virus during the corresponding IAP; 3) the standard errors of virus titer and the proportion of plants infected during the corresponding IAP; and 4) the standard errors of virus titer and the proportion of sucrose sachets containing virus during the corresponding IAP. Neither TYLCV nor ToMoV-A titers were significantly correlated with the proportion of infected IAP plants (TYLCV: N=15, F=0.85, Coeff=68.50, *P*=0.3746, R^2=0.0611; ToMoV: N=9, F=0.03, Coeff=95.02, *P*=0.8727, R^2=0.0039) or sucrose sachets containing virus or viral DNA (TYLCV: N=15, F=2.52, Coeff=64.68, *P*=0.1361, R^2=0.1626; ToMoV: N=9, F=0.48, Coeff=92.07, *P*=0.5086, R^2=0.0648). However, the standard error of TYLCV titer was significantly positively correlated with the proportion of infected IAP plants (N=15, F=21.93, Coeff=73.09, *P*=0.0004, R^2=0.6278) and sucrose sachets containing virus (N=15, F=6.96, Coeff=96.70, *P*=0.0205, R^2=0.3485). Although standard error of ToMoV-A titer was not significantly correlated with infected proportion of IAP plants (N=5, F=3.10, Coeff=75.55, *P*=0.1766, R^2=0.5081), R-square value showed there was a medium correlation between them. There was no significant correlation between standard error of ToMoV-A titer and proportion of sucrose sachets containing virus (N=5, F=0.14, Coeff=105.22, *P*=0.7294, R^2=0.0459).

Symptom expression and height of tomato plants

Symptoms were monitored in all rounds but data are only presented for Rounds 0-2. Beginning in Round 3, the number of insecticide applications made to plants after the IAP to kill immature whiteflies was increased from one application to two applications made one week apart. This caused phytotoxicity symptoms that included twisting, elongation of internodes, and foliar strapping that interfered with symptom scoring and height measurements for virus

infections. In addition, data from control plants in rounds 1-2 were not included in analyses due to spider mite infestation and water logging, but they were confirmed to be virus-free using PCR. The analyses of symptoms are reported for 'healthy plants'; this includes control plants in Round 0, and plants that did not become infected with viruses during the IAP (confirmed using PCR) in Rounds 0-2.

First, an ANOVA was conducted to compare heights of healthy, TYLCV, and ToMoV infected plants during each weekly evaluation for each round (Table 2.5). The heights of healthy, TYLCV and ToMoV plants were significantly different at each evaluation period during Round 0. Heights during Round 1 and Round 2 were only significantly different 28dpi.

Another analysis was conducted to compare total plant growth between 7 and 28dpi (Table 2.6). Plant growth was significantly different among rounds for both viruses, and the average plant growth of virus infected plants increased throughout the course of the experiments. There are no significant differences in plant growth among rounds for healthy plants. In the first analysis, differences in plant height were compared among healthy, TYLCV, and ToMoV infected plants during each round. In Round 0, healthy plants grew significantly more than virus infected plants, and ToMoV infected plants grew significantly taller than TYLCV infected plants. There were no differences in plant growth between ToMoV and healthy plants in Round 1, but growth of TYLCV infected plants was significantly less from both healthy and ToMoV infected plants. In Round 2, healthy plants grew significantly more than TYLCV infected plants, but differences in plant growth were not significantly different between ToMoV infected and healthy plants, or ToMoV and TYLCV infected plants.

The same aforementioned analyses were conducted to examine differences in symptoms. First, differences in symptoms among healthy, TYLCV, and ToMoV infected plants were

compared for each evaluation date and round of transmission (Table 2.5). Virus infected plants always showed significantly higher symptoms 14dpi. During Round 0, ToMoV-infected plants exhibited symptoms beginning 7dpi, and symptom ratings were significantly higher than TYLCV-infected plants 7, 14, 21 and 28dpi. Symptoms observed during Round 1 and Round 2 were significantly different between both viruses 21 and 28dpi only, and symptoms were higher for TYLCV in Round 2 only. Next, two analyses of the differences in symptom development were conducted to 1) compare differences in symptom development of TYLCV and ToMoV during each round, and 2) compare differences in symptom development of each virus among rounds (Table 2.6). Significant differences in symptom development between viruses and healthy plants was observed for Rounds 0-2, but only between viruses in Round 2. There were also significant differences for each virus among rounds. Symptoms of TYLCV were significantly more severe in Round 0, but no differences in symptom ratings were observed in Rounds 1 and 2. ToMoV symptom ratings were significantly higher in Rounds 0 and 1, than Round 2, and there were no differences between symptoms in Rounds 0 and 1.

Discussion

There was no evidence for strong selection by the plant or whitefly vectors in this experiment, and these results were observed in each of the three independently run bio-replications for both viruses. When examining the transmission efficiency of individual whiteflies to sucrose sachets, ToMoV-A transmission is always low, even when 80% of IAP plants are infected with ToMoV. TYLCV transmission to sucrose sachets decreases through time and is similar to decreases in transmission to plants. These results suggest there was no adaptation of either virus to *B. tabaci* in this study. There are also no consistent changes in viral

titers measured 28dpi through time. The titers of ToMoV-A and ToMoV-B fluctuate through time, and statistically similar titers were observed during Round 0 and Round 2 even though there was ~3-fold greater transmission in Round 0. There were no significant differences in titers of TYLCV infected plants throughout the experiment, but titers were numerically higher in Rounds 3-4 than Rounds 0-1. The proportion of infected plants and severity of symptoms decreases through time for both viruses, and symptoms of TYLCV appear earlier in Rounds 1-2 than Round 0. Overall, fitness of both viruses decreased through time.

A recently published serial transmission study on a persistent and circulative aphid transmitted ssRNA Luteovirus provided evidence for adaptation of SbDV in pea, but not to soybean or the aphid vectors (Tian et al. 2017). In their study, there were eight serial transmissions in pea, whereas in the two bio-replicates of SbDV on soybean, transmission was lost after six and seven serial passages. In our or the current study, transmission of ToMoV was lost after two serial passages, and TYLCV transmission had declined to 27% of plants infected after the third passage. It is possible that whitfefly-plant-virus interactions during the AAP contributed to the decreases in transmission over time if whiteflies preferred to feed on healthier plant tissue. Over time, the variation in symptom rating and titers among AAP plants increased. We did not specifically test for the presence of the virus or virus titers in whiteflies; however, if virus titer influences host plant probing or settling cues, viral loading of the vector, or phloem feeding, then it is possible that acquisition of the virus could have changed over time. Small changes in behaviors associated with whitefly feeding would more strongly impact a bipartite virus like ToMoV than a monopartite virus like TYLCV because two virus particles (containing ToMoV-A and ToMoV-B) must co-infect a cell, and in this study sucrose sachet data suggests fewer whiteflies are responsible for transmitting ToMoV. Titers of some source plants have also

been reported to be correlated with gemnivirus transmission efficiency by *B. tabaci* (Wintermantel et al. 2016), and even when virus population size is large, viral load could be limited by barriers caused by host, vector or virus (Gutiérrez et al. 2012). In this study, there was not a significant correlation between virus titer and transmission efficiency; however, we did find positive correlation between standard error of virus titer and transmission efficiency of TYLCV, which also suggests that plants used in the AAPs may have been variable in quality for their whitefly vectors, which may have influenced transmission outcomes. Studies examining vector-plant interactions on healthy and virus infected plants have reported both increases and decreases in plant attractiveness and quality for MEAM1 *B. tabaci*, and in some studies, whiteflies tended to feed on healthy tomato plants more than plants infected with *Tomato chlorosis virus* or *Tomato severe rugose virus* because of low plant quality caused by virus infection (Maluta et al. 2017). Other plant-related factors that have been shown to influence transmission efficiency include plant species, leaf size, quality of source plants, viral load in vector, and plant growing conditions.

Other possible explanations of the loss of fitness in both TYLCV and ToMoV over time may be related to the genetic diversity of the virus clones used in this study, selection, bottlenecks and drift. The viral genetic diversity of the source populations is going to influence adaptive potential of these populations, and the absence of specific genotypes may hinder adaptation until they arise in the population due to mutation that occurs in the laboratory. Mutation is one of the forces of virus evolution that can help to increase diversity needed to adapt to new environments (Roossinck 1997, Tian et al. 2017). Virus populations frequently endure genetic bottleneck events during within-host progression as well as host-to-host transmission (Pfeiffer and Kirkegaard 2006, McCrone and Lauring 2018), and both plant

infection and whitefly transmission may have reduced genetic diversity through selection, especially in these isolated populations after repeated exposure. In this experiment, selection of TYLCV and ToMoV populations for host plant adaptation might decrease their transmissibility by whiteflies through time. Intrinsic decay rates of virions and host defenses can be factors causing bottlenecks too (Gutiérrez et al. 2012), and tomatoes infected with ToMoV have been reported to undergo recovery (Rajabu et al. 2018). Random effects and genetic drift might counteract selection, which does not efficiently fix beneficial mutations or purge deleterious ones in small populations (Robertson 1960, McCrone and Lauring 2018). Genetic sequencing of these populations and an examination of genetic diversity through time would provide information about the relative effects of mutation, selection, drift and bottlenecks on these populations.

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Table 2.1. Primer information.

Primer name	Sequence (5'-3')	Species	Expected size (bp)	References
Btab-Uni F	GAGGCTGGAAAATTAGAAGTATTTGG	*Bemisia tabaci*	795	Shatters et al. 2009
Btab-Uni R	CTTAAATTTACTGCACTTTCTGCCACATTAG			
TYLCV convFor	CCTCTGGCTGTGTTCTGTTATC	TYLCV	257	
TYLCV convRev	GCAATCTTCGTCACCCTCTAC			Rajabu et al 2018
TomoV-convFor	GTCCAATACTCTCTCGTCCAATC	TOMOV-A component	239	
TomoV-convRev	CAGCGGCCTTGTTAATTCTTG			Rajabu et al 2018
ToMoV-B-Fw	AAGCCCAAGTCTGGACATGG	TOMOV-B component	205	
ToMoV-B-Rv	TCATCAACGGACCCACTTCG			This study

Table 2.2. Age of acquisition access period plants used to successfully transmit virusin the serial transmission experiments.

Round	TYLCV	ToMoV
0	90dpi[2]	90dpi
1	30dpi	30dpi
2	30dpi	60dpi
3	30dpi	-[1]

[1]No plants because transmission from previous round was not successful.
[2]Days post inoculation.

Table 2.3. The mean(standard error) virus titer of *Tomato yellow leaf curl virus* (TYLCV) and *Tomato mottle virus* (ToMoV)A and B components 28 days post inoculation (dpi) for each round of transmission.

	TYLCV		ToMoV		
Round	N[1]	Titer[2]	N	ToMoV-A Titer	ToMoV-B Titer
0	8	9193523(2353705)a	8	5705649(1943047)a	5792931(1771917)a
1	9	4623986(2210920)a	9	5998623(1820173)a	3401356(1659865)a
2	9	8898683(2210920)a	9	4056770(2526293)a	3589485(2303794)a
3	9	7633094(2210920)a	-[5]	-[5]	-[5]
P-value[4,6]		3, 29, F=0.87, *P*=0.4675		2, 17, F=0.21, *P*=0.8150	2, 17, F=0.55, *P*=0.5853

[1] Number of virus infected plants tested virus titer at 28dpi in each round.

[2] Units of titer is virus copy number per ng total DNA.

[3]ToMoV-A component.

[4]Mean comparison of titers were conducted for each virus across rounds.

[5]Data was not available because there was no successful transmission to Round 3.

[6] Num DF, Den DF, F Value, Pr>F. Not statistically significant (*P*>0.05).

Table 2.4. The mean(standard error) virus titer of acquisition access period (AAP) plants, the proportion of plants infected, and proportion of sucrose sachets containing virus after being fed upon by putatively viruliferous whiteflies during each round of serial transmission.

				Virus titer[1]		Plant[2]		Sucrose sachets	
	Round	N[3]	DPI	DNA-A		N	Proportion infected	N	Proportion infected
TYLCV	0	8	90	4132749(2133658)a[4]		30	0.93(0.04)a	-[7]	-[7]
	1	9	30	4623986(2004223)a		30	1.00(0.00)a	60	0.77(0.05)a
	2	9	30	8898683(2004223)a		30	0.53(0.09)b	60	0.42(0.06)b
	3	9	30	7633094(2004223)a		30	0.67(0.09)b	60	0.20(0.05)bc
	4	-[5]	-[5]	-[5]		30	0.27(0.08)c	60	0.13(0.04)c
	P-value			3, 29, F=1.28, *P*=0.2990			4, 145, F=5.34, *P*=0.0005***		3, 236, F=17.16, *P*<.0001***
				Virus titer		Plant		Sucrose sachets	
	Round	N	DPI	DNA-A	DNA-B	N	Proportion infected	N	Proportion infected
ToMoV	0	9	90	2039281(1007847)b	1566582(833369)a	30	1.00(0.00)a	-	-
	1	9	30	5998623(1007847)a	3401356(833369)a	30	0.80(0.07)b	60	0.02(0.02)a
	2	9	60	1417939(1007847)b	2267444(833369)a	30	0.33(0.09)c	60	0.08(0.04)a
	3	9	107	-[5]	-[5]	-[5]	-[5]	-[5]	-[5]
	P-value[6]			2, 22, F=6.08, *P*=0.0079**	2, 22, F=1.23, *P*=0.3104		2, 86, F=6.03, *P*=0.0035**		2, 177, F=1.47, *P*=0.2335

[1]Units of titer is virus copy number per ng total DNA.
[2] Plants from Round 0 are infected by agro-inoculation and plants after Round 1 are infected by whitefly transmission Number of virus infected AAP plants tested for virus titer in each round.
[3]Number of observations.
[4] Mean separation corresponds to the values presented in each column; separate analyses were conducted to examine titer, proportion of plants infected, and virus in sucrose sachets for each virus.
[5] Data was not available due to loss of transmission.
[6] Num DF, Den DF, F Value, Pr>F. Not significant (*P*>0.05) or significant at *P*<0.05(*), 0.01(**) or 0.001(***).
[7]Whiteflies infected sucrose sachets and IAP plants at the same time and sucrose sachets were testing began Round 1.

Table 2.5. The mean(standard error) of height and symptom severity score of tomato plants 7, 14, 21 and 28 days post inoculation(dpi) during Round 0, Round 1 and Round 2.

Round	Attribute	DPI	Healthy[1]	TYLCV	ToMoV	P-value[2]
0	Height(cm)[31]	7	3.96(0.10)a	3.60(0.10)b	3.82(0.10)ab	2, 86, F=3.54, *P*=0.0332*
		14	4.81(0.13)a	4.00(0.13)b	4.53(0.13)a	2, 86, F=10.21, *P*=0.0001***
		21	6.04(0.17)a	4.24(0.17)c	5.26(0.17)b	2, 86, F=28.08, *P<.0001***
		28	7.37(0.19)a	4.45(0.19)c	5.91(0.19)b	2, 86, F=59.76, *P<.0001***
	Symptom[4]	7	1(0.07)b	1(0.07)b	1.52(0.07)a	2, 86, F=16.99, *P<.0001***
		14	1(0.07)c	1.5(0.07)b	2.76(0.07)a	2, 86, F=140.08, *P<.0001***
		21	1(0.07)c	2.73(0.07)b	3.79(0.07)a	2, 86, F=400.60, *P<.0001***
		28	1(0.05)c	3.7(0.05)b	3.93(0.05)a	2, 86, F=833.55, *P<.0001***
1	Height(cm)	7	3.10(0.55)a	3.62(0.14)a	3.61(0.14)a	2, 57, F=0.43, *P*=0.6530
		14	4.00(0.69)a	4.17(0.18)a	4.48(0.18)a	2, 57, F=0.87, *P*=0.4257
		21	5.70(0.90)a	4.96(0.23)a	5.64(0.24)a	2, 57, F=2.13, *P=0.1281*
		28	7.25(1.00)a	5.71(0.26)c	6.71(0.27)b	2, 57, F=4.13, *P=0.0211**
	Symptom	7	1.00(0.31)a	1.10(0.08)a	1.32(0.08)a	2, 57, F=2.09, *P*=0.1330
		14	1.00(0.36)b	2.70(0.10)a	2.89(0.10)a	2, 57, F=12.85, *P<.0001***
		21	1.00(0.36)c	2.90(0.10)b	3.71(0.10)a	2, 57, F=39.09, *P<.0001***
		28	1.00(0.43)c	3.30(0.11)b	3.89(0.11)a	2, 57, F=24.48, *P<.0001***
2	Height(cm)	7	2.85(0.10)a	3.03(0.14)a	2.82(0.17)a	2, 57, F=0.69, *P*=0.5069
		14	3.71(0.13)a	3.67(0.19)a	3.74(0.24)a	2, 57, F=0.02, *P*=0.9757
		21	5.60(0.16)a	5.02(0.23)a	5.74(0.29)a	2, 57, F=2.64, *P*=0.0804
		28	6.67(0.18)a	5.70(0.27)b	6.43(0.34)ab	2, 57, F=4.38, *P*=0.017*
	Symptom	7	1.00(0.05)b	1.19(0.08)ab	1.4(0.10)a	2, 57, F=7.93, *P*=0.0009***
		14	1.00(0.07)b	1.62(0.11)a	1.4(0.13)a	2, 57, F=12.90, *P<.0001***
		21	1.00(0.07)c	2.69(0.10)a	1.6(0.13)b	2, 57, F=76.99, *P<.0001***
		28	1.00(0.14)c	28.75(0.20)a	2.10(0.26)b	2, 57, F=29.74, *P<.0001***

[1] Include control and non-infected tomatoes from virus transmission attempts.
[2] Num DF, Den DF, F Value, Pr>F. Not significant (*P*>0.05) or significant at *P*<0.05(*), 0.01(**) or 0.001(***).
[3]Height was measured from the base of the tomato plant at the soil line to the apical meristem.
[4] Virus symptom severity rating on a scale of 1-4: 1=No symptom, 4=severe symptom.

Table 2.6. The mean(standard error) in the difference of height and symptom severity score of tomato plants from 7 to 28 days post inoculation (dpi) during Round 0, Round 1 and Round 2.

Attribute	Round	Healthy[4]	TYLCV	ToMoV	P-value[3]
Height(cm)[1]	0	3.4(0.16)	0.85(0.16)	2.09(0.16)	2, 86, F=62.61, P<.0001***
	1	4.15(0.67)	2.09(0.17)	3.10(0.18)	2, 57, F=10.73, P=0.0001***
	2	3.82(0.18)	2.67(0.26)	3.60(0.33)	2, 57, F=6.60, P=0.0026**
	P-value[3]	2, 63, F=1.68, P=0.1948	2, 73, F=28.88, P<.0001***	2, 64, F=11.09, P<.0001***	
Symptom[2]	0	0.00(0.10)	2.70(0.10)	2.41(0.10)	2, 86, F=264.99, P<.0001***
	1	0.00(0.51)	2.20(0.13)	2.57(0.14)	2, 57, F=12.44, P<.0001***
	2	0.00(0.15)	1.68(0.23)	0.70(0.29)	2, 57, F=18.70, P<.0001***
	P-value[3]	-	2, 73, F=6.92, P=0.0018**	2, 64, F=22.12, P<.0001***	

[1]Height was measured from the base of the tomato plant at the soil line to the apical meristem.
[2]Virus symptom severity rating on a scale of 1-4: 1=No symptom, 4=severe symptom.
[3] Num DF, Den DF, F Value, Pr>F. Not significant ($P>0.05$) or significant at $P<0.05(^{*})$, $0.01(^{**})$ or $0.001(^{***})$. Separate analyses were conducted to compare among virus infected and healthy plants during each round, and to examine differences in heights among plants from each treatment across different rounds.
[4]Includes control and non-infected tomatoes from virus transmission attempts.

Figure 2.1. Inoculation access period cages modified to enable controlled whitefly release and provide air flow to contents.

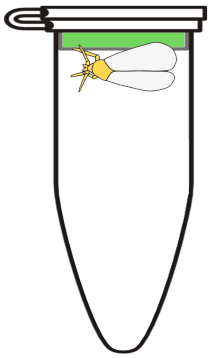
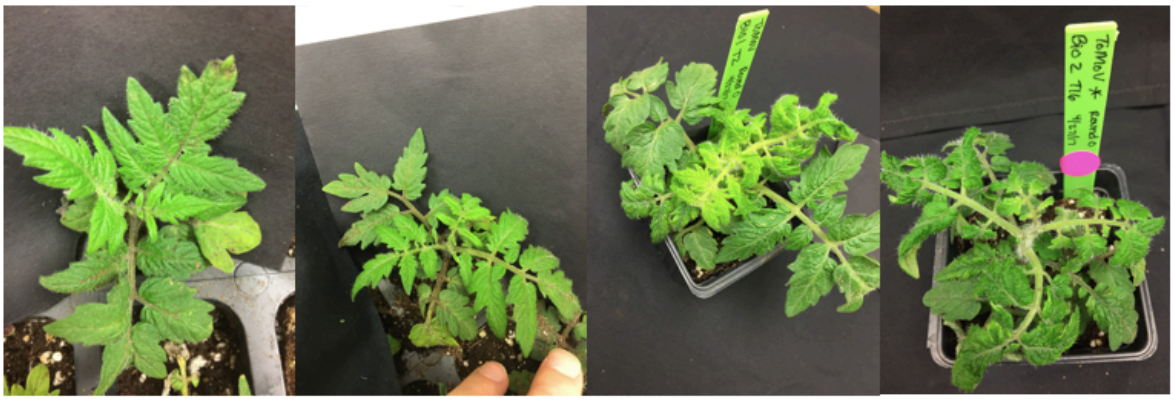


Figure 2.2. Sucrose sachet. A volume of 16µl 15% sucrose solution was added on one layer of parafilm on lid of 1.7ml tube and covered by another layer of parafilm. A single whitefly was released into each tube and allowed to feed on sucrose solution through parafilm for 48 hours. Transmission to sucrose sachets was tested concurrent with inoculation access period for transmission to tomato plants.

Tomato yellow leaf curl virus (TYLCV)

1 2 3 4

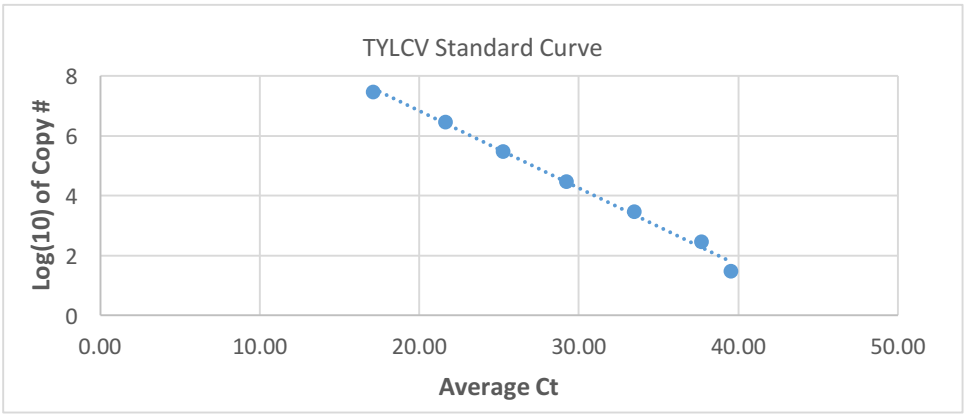
Tomato mottle virus (ToMoV)



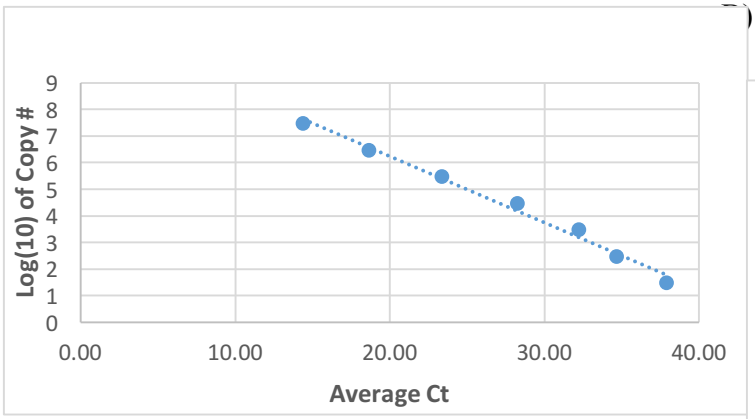
1 2 3 4

Figure 2.3. The 1-4 symptom scale used to visually assess symptoms resulting from infectionby *Tomato yellow leaf curl virus* (TYLCV) and *Tomato mottle virus* (ToMoV). Symptom severity was rated at 7, 14, 21 and 28days post inoculation from Round 0 to Round 2.

Tomato yellow leaf curl virus (TYLCV)



Tomato mottle virus A component (ToMoV-A)



Tomato mottle virus B component (ToMoV-B)

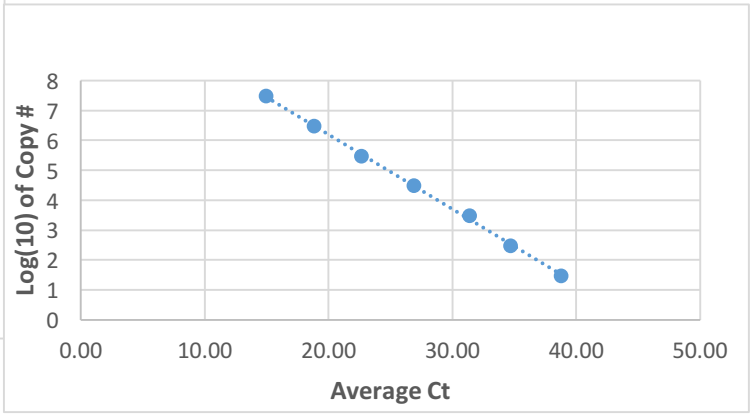


Figure 2.4. Standard curve of TYLCV, ToMoV-A and ToMoV-B from qPCR. Genomic viral DNA is present at 30000000 copies, 3000000 copies, 300000 copies, 30000 copies, 3000 copies, 300 copies and 30 copies.

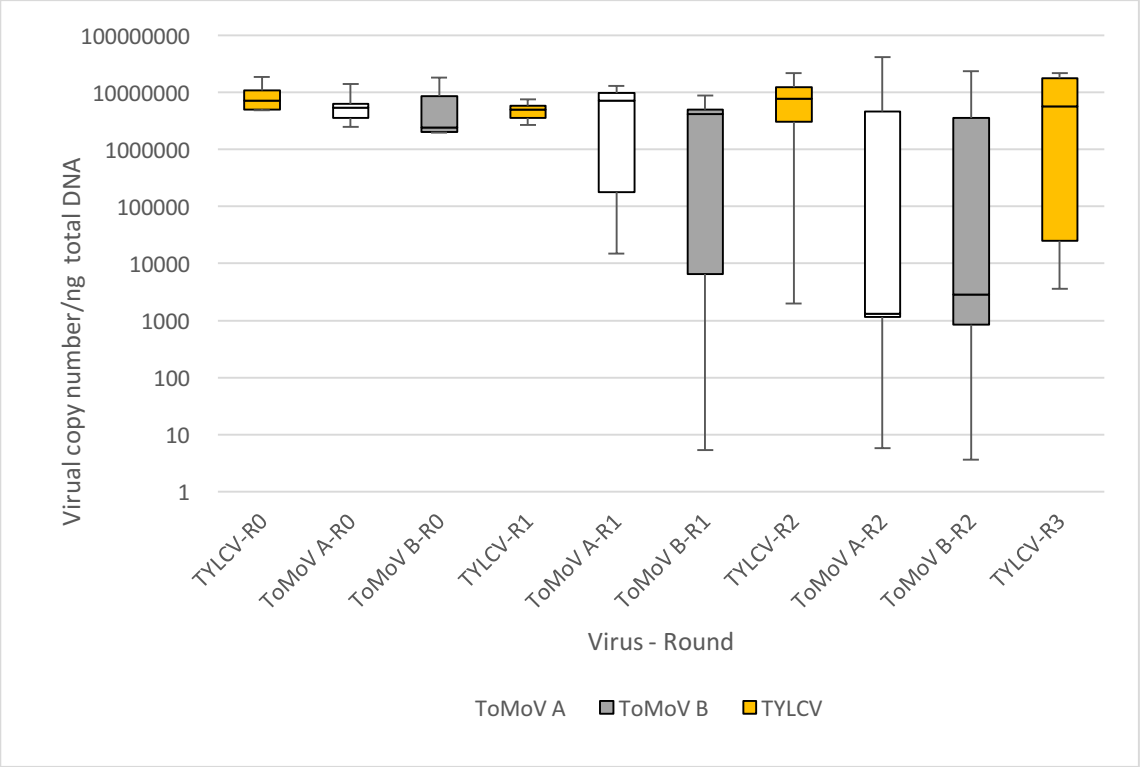


Figure 2.5. Box plot of virus titer of *Tomato yellow leaf curl virus* (TYLCV) and *Tomato mottle virus* (ToMoV) (A and B components) at 28 days post inoculation during each round of transmission: R0=Round 0, R1=Round 1, R2=Round 2, R3=Round 3.

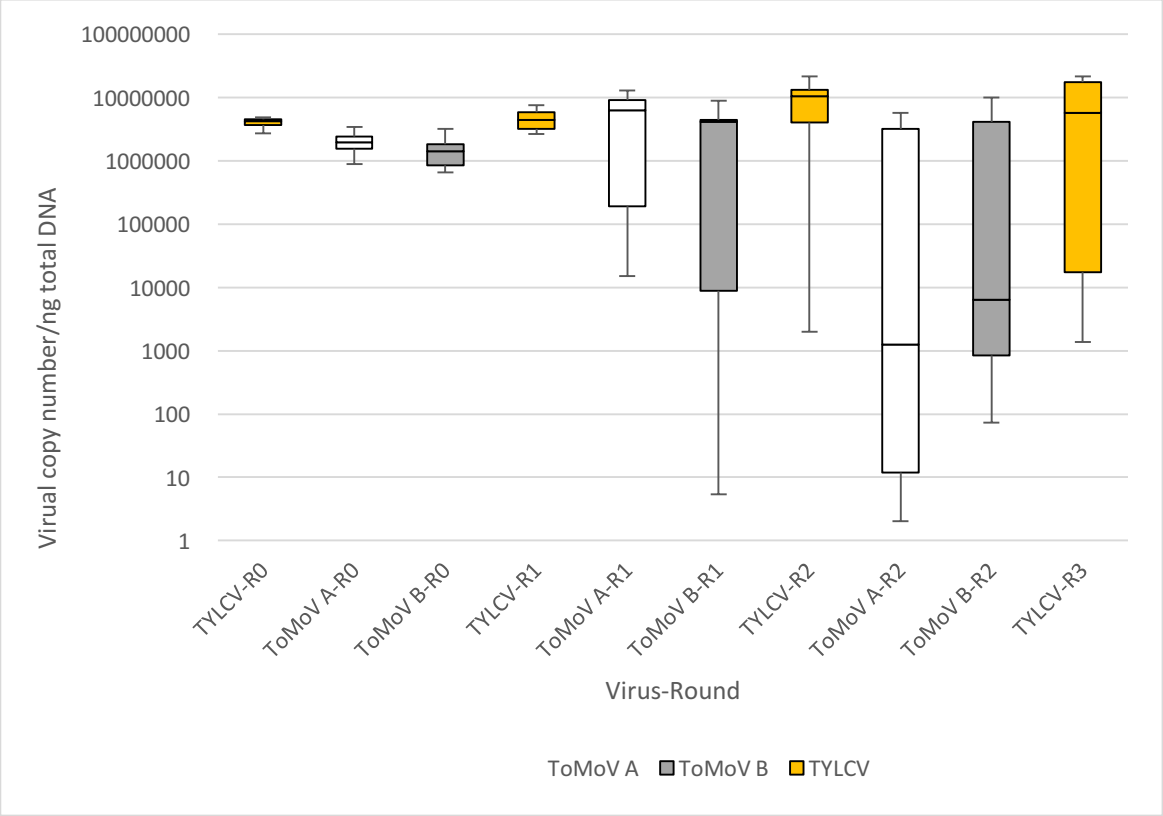


Figure 2.6. Box plot of virus titer of *Tomato yellow leaf curl virus* (TYLCV) and *Tomato mottle virus* (ToMoV) (A and B components) used as acquisition access period plants for each round of transmission: R0=Round 0, R1=Round 1, R2=Round 2, R3=Round 3.

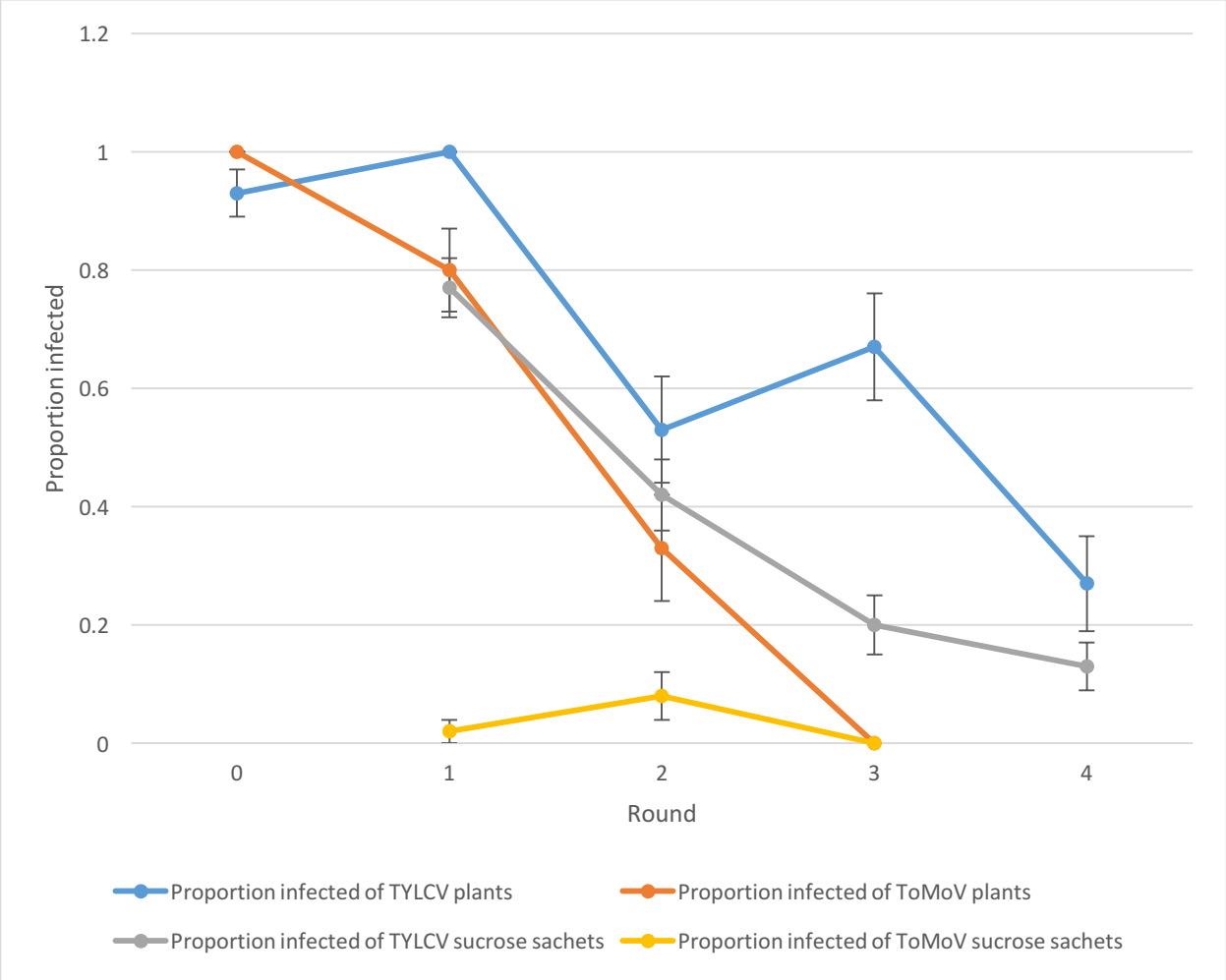


Figure 2.7. Proportion of infected tomato plants and sucrose sachets containing virus during rounds of serial transmissions.

Chapter Three

Investigating virus titer distributions in *Tomato yellow leaf curl virus* and *Tomato mottle virus* infected plants through time

Introduction

Begomoviruses, the largest genus within family Geminiviridae (Varsani et al. 2014, Rosario et al. 2015) with at least 322 species (ICTV 2017), cause enormous economic and crop production damage worldwide (Polston and Anderson 1995, Navas-Castillo et al. 2011), especially on tomato plants (Polston 1993, Polston et al. 1996, 1999, Momol et al. 1999). Two economically important tomato viruses in the U.S. are *Tomato yellow leaf curl virus* (TYLCV) and *Tomato mottle virus* (ToMoV). Both species belong to the genus, *Begomovirus.* These viruses are transmitted in a persistent and circulative manner (Czosnek et al. n.d., Ghanim 2014, Rosen et al. 2015) by the whitefly, *Bemisia tabaci* Gennadius (Hemiptera: Aleyrodidae), one of the world's 100 worst invasive species (Lowe et al. 2004),.

Begomovirus DNA genomes can be monopartite (TYLCV) or bipartite (ToMoV) consisting of A and B components packaged into separate particles (Hitefly et al. 1999, Zhang et al. 2001). The genome of A component of bipartite viruses and monopartite viruses encode coat protein (CP), replication-associated protein (Rep) as well as replication enhancer protein (REn),

transcriptional activator protein (TrAP), virulence factor (C4 or AC4), and V2 or AV2 protein. Nuclear shuttle protein (NSP) and movement protein (MP) are encoded by genome of B component of bipartite viruses (Gutierrez 1999, Hitefly et al. 1999, Rosario et al. 2015) (Figure 1). Rep can interact with REn (Settlage et al. 2005) for viral replication (Taylor et al. 1999). C4 or AC4 plays an important role in symptom development, induces cell proliferation (Latham et al. 1997), and counteracts post–transcriptional gene silencing (PTGS) (Carvalho et al. 2008, Hanley-Bowdoin et al. 2013). V2 and AV2 proteins can encode a suppressor of PTGS to overcome a defense mechanism of host plants (Glick, Zrachya, et al. 2009, Zhang et al. 2012, Hanley-Bowdoin et al. 2013). In bipartite begomoviruses, NSP and MP are responsible for movement of viral DNA from one cell to the next cell and through the phloem (Noueiry et al. 1994, Sanderfoot and Lazarowitz 1995, Hanley-Bowdoin et al. 2013). CP functions as NSP in monopartite begomoviruses (Poornima Priyadarshini et al. 2011). Accumulation and movement of monopartite begomoviruses is solely DNA-A dependent, whereas bipartite begomoviruses are DNA-A and DNA-B dependent (Liu et al. 1997).

'Florida Lanai' tomato (*Solanum lycopersicum* L.) is a known susceptible variety for studies on TYLCV and ToMoV infection (Rajabu et al. 2018). Previous studies in Florida Lanai have documented titers of DNA-A of TYLCV and ToMoV at three time points after agroinoculation up to 31 days post inoculation (dpi) (Rajabu et al. 2018). However, differences of virus titers were not examined after whitefly transmission, at different leaf positions of plants, ToMoV DNA-B component (ToMoV-B) titers were not quantified, and titers after 31dpi are not known. This study was conducted to further understand virus titer accumulation throughout infected plants at different time points after inoculation. Our specific objectives were to compare

virus titers among three leaf positions, and at four time points up to 69dpi, and examine the relationship between titers and symptom severity.

Materials and Methods

Virus infected Lanai tomato plants

The protocols and methods for whitefly colony maintenance, growing Lanai tomatoes, agroinoculation, DNA extraction, virus quantification, and symptom monitoring are the same as those used in Chapter Two (pages 36-43). In this experiment, leaf tissue was sampled and symptoms were scored at 21, 28, 35 and 69dpi. A total of six leaf discs were taken per plant: two leaf discs were taken from the 1st, 3rd, and 5th fully expanded leaf (counted from the apex to the bottom of tomato plants) and placed separately into three different 2ml tubes with 2.3mm diameter stainless steel ball (BioSpec Products, Inc., Bartlesville, OK). Samples were immediately flash frozen in liquid nitrogen and stored at -80℃ Revco UxF freezer (Thermo Fisher Scientific Inc., Waltham, MA) until DNA extraction. Leaf samples were taken from the same plants at each time point; even though samples destroyed leaf tissue, the same leaves were never sampled twice due to plant growth. Virus titers were measured on two different cohorts of plants. The first cohort consisted of 7 TYLCV infected plants and 7 ToMoV infected plants from Round 2 that were inoculated using whitefly transmission. The second cohort included 5 TYLCV infected plants and 5 ToMoV infected plants that were infected using agroinoculations.

Statistical analysis

Statistical analysis to compare titers through time was performed using repeated measures ANOVA implemented in SAS PROC GLIMMIX version 9.4 (SAS Institute, Cary, NC).

Separate analyses were conducted for TYLCV, ToMoV-A, ToMoV-B, and for each method of inoculation (whitefly and agroinoculation). Titers of plants were examined using dpi, leaf position, and their interaction term as main effects. A regression analysis was also performed to test for correlations between virus titer and symptoms. *P*-values <0.05 were considered statistically significant.

Results

Accumulation of virus titer in Lanai tomato plants

The averages, standard errors, and significance values for the main effects dpi and leaf position are provided in Table 3.1 and Table 3.2, respectively. There were significant differences of average TYLCV titer across different time points for both whitefly and agroinoculated plants (Table 3.1, Figure 3.1). Overall, the average virus titers in plants increased between 21-28dpi, decreased between 28-35dpi, and then increased again between 35-69dpi, but not all increases and decreases were statistically significant. The only exception to this was observed in whitefly inoculated plants where titers decreased between 21-28dpi, slightly increased between 28-35dpi, but then increased significantly between 35-69dpi and was the highest titer observed among all observations. Virus titers in the 1st, 3rd, 5th leaf positions (from apex to bottom) in tomato plants were not significantly different, except TYLCV plants infected by agroinoculation (Table 3.2, Figure 3.2). Although we cannot directly compare whitefly and agroinoculated plants statistically because they were not conducted at the same time, the lowest virus titers were observed in ToMoV infected plants inoculated with whiteflies. In all of the other virus-inoculation combinations, the titers were numerically higher in the 5th leaf, followed by the 3rd and then 1st,

and these differences were statistically significant in the TYLCV agroinoculated plants. The interaction term of dpi and leaf position were not statistically significant for agroinoculated TYLCV (6, 47, F=0.88, *P*=0.5188), agroinoculated ToMoV-A (6, 48, F=1.21, *P*=0.3183) or ToMoV-B (6, 48, F=0.68, *P*=0.6650), whitefly transmitted ToMoV-A (6, 72, F=1.08, *P*=0.3842) or ToMoV-B (6, 72, F=1.03, *P*=0.4120), but was significant for whitefly transmitted TYLCV (6, 70, F=3.66, *P*=0.0032).

Symptom expression and virus titer

A regression was performed to examine the relationship between symptom expression and virus titer. Overall, symptom expression of tomato plants was significantly positively correlated with virus titer of TYLCV infected by agroinoculation (N=20, Coeff=51.96, *P*=0.004, R^2=0.3772) and whiteflies (N=28, Coeff=108.80, *P*=0.0002, R^2=0.411), and virus titer of ToMoV-A (N=28, Coeff=345.55, *P*=0.0009, R^2=0.3511) and ToMoV-B (N=28, Coeff=270.88, *P*<.0001, R^2=0.461) infected by whiteflies. However, data of ToMoV-A and B components from agro-inoculation were not analyzed because all symptom severity scores were the same. The other regression was used to test correlation between ToMoV-A and ToMoV-B titers. Virus titers of ToMoV-A and ToMoV-B were significantly positively correlated with each other in plants infected by agroinoculation (N=20, Coeff=36.62, *P*<.0001, R^2=0.5947) and whitefly transmission (N=28, Coeff=282.40, *P*<.0001, R^2=0.5666).

Discussion

This experiment was conducted to examine within-plant virus titers and monitor changes in these titers through time. The patterns of increases and decreases in the average plant virus titer

through time observed in this study are similar to those reported by Rajabu et al. (2018), except the peaks in virus titer occur later in this study. Peak titers were reported previously to occur 24dpi in TYLCV and 17dpi in ToMoV agroinoculated Florida Lanai tomato plants, whereas in this study they generally occur at 28dpi for both viruses. In agroinoculated ToMoV and TYLCV plants, there was a significant increase in titers observed 21-28dpi followed by a significant decrease 28-35dpi. In previous studies, reduced virus accumulation and recovery of plants has been universally considered as a consequence of natural defense mechanism in plants induced by viruses (Ratcliff et al. 1999, Zhou et al. 2008, Ma et al. 2015, Rajabu et al. 2018), and a major antiviral defense mechanism in plants is mediated by gene silencing (Ratcliff et al. 1999). It has also been shown that viruses can infect plants by suppressing plant RNA silencing defenses (Qu and Morris 2005, Pumplin and Voinnet 2013), and this has been reported for begomoviruses (Hanley-Bowdoin et al. 2013). In the study by Rajabu et al. (2018) they hypothesized that plant defense mechanisms may be responsible for observed decreases in titer, but their study was terminated after 31 days. In this study, reductions in virus titer were followed by significant increases between 35-69 days in agroinoculated plants, and numerical increases were observed in whitefly transmitted infections. Future studies are needed to further investigate the factors influencing virus titer fluctuations in plants through time.

Different leaf positions were also sampled, but titers were not significantly different among leaf positions in this study before 35dpi. In a study on *Tomato yellow leaf curl Sardinia virus,* there were no significant differences in virus titer in first, second, third or fourth leaves from the apex, but titers were numerically higher in the fourth leaf (Mason et al. 2008). Although not significant, virus titers were generally higher in the 5^{th} leaf position in this study, which would be the closest to original inoculation site for both whitefly transmission and agroinoculation. There

was also a positive correlation between symptom expression and virus titer of TYLCV and ToMoV in this experiment. Higher virus titer lead to more severe symptoms in plants.

In the future this experiment should be repeated to further examine changes in virus titer after whitefly transmission. Titers of TYLCV infected plants showed slightly different patterns of increase, and reached very high levels 69dpi, but more replication of this experiment is needed to confirm these results. Overall, this study provides important information about changes in virus titer after agroinoculation and whitefly transmission that can be used to assist with future studies on TYLCV and ToMoV.

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Table 3.1. The mean (standard error) of virus titer of *Tomato yellow leaf curl virus* (TYLCV), *Tomato mottle virus* A component (ToMoV-A) and *Tomato mottle virus* B component (ToMoV-B) in Lanai tomato plants 21, 28, 35 and 69 days post inoculation (dpi).

	TYLCV[1]		ToMoV-A[1]		ToMoV-B[1]	
	Whitefly transmission	Agroinoculation	Whitefly transmission	Agroinoculation	Whitefly transmission	Agroinoculation
21DPI	2551391(1357390)b	1373842(663117)c	219(1023947)a	1824594(478135)b	139(931492)a	3971096(636325)a
28DPI	1199919(1357390)b	6082977(637102)a	1971454(1023947)a	4479256(478135)a	1099508(931492)a	4351722(636325)a
35DPI	1519738(1430815)b	3679100(637102)bc	103(1023947)a	1861410(478135)b	1(931492)a	2296714(636325)a
69DPI	11562620(1357390)a	5739621(637102)ab	584813(1023947)a	4238902(478135)a	1731190(931492)a	4235779(636325)a
P-value[2]	3, 70, F=13.09, *P*<.0001***	3, 47, F=11.12, *P*<.0001***	3, 72, F=0.82, *P*=0.4844	3, 48, F=9.27, *P*<.0001***	3, 72, F=0.85, *P*=0.4732	3, 48, F=2.27, *P*=0.0927

[1] Units of titer is virus copy number per ng total DNA.

[2] Num DF, Den DF, F Value, Pr>F. Not significant ($P>0.05$) or significant at $P<0.05(^{*})$, $0.01(^{**})$ or $0.001(^{***})$. Significance values are listed by column.

Table 3.2. The mean (standard error) of virus titer of *Tomato yellow leaf curl virus* (TYLCV), *Tomato mottle virus* A component (ToMoV-A) and *Tomato mottle virus* B component (ToMoV-B) in the 1st, 3rd and 5th leaf in Lanai tomato plants.

	TYLCV[1]		ToMoV-A[1]		ToMoV-B[1]	
Leaf location	Whitefly transmission	Agroinoculation	Whitefly transmission	Agroinoculation	Whitefly transmission	Agroinoculation
1st leaf	3628415(1199775)a	3083502(568727)b	1495444(886764)a	2660108(414077)a	960844(806696)a	2869299(551074)a
3rd leaf	4471677(1199775)a	3962822(551747)ab	416265(886764)a	3255129(414077)a	1126302(806696)a	3903473(551074)a
5th leaf	4525158(1199775)a	5610331(551747)a	5732(886764)a	3387885(414077)a	35983(806696)a	4368711(551074)a
P-value[2]	2, 70, F=0.18, *P*=0.8380	2, 47, F=5.28, *P*=0.0086**	2, 72, F=0.75, *P*=0.4747	2, 48, F=0.88, *P*=0.4229	2, 72, F=0.53, *P*=0.5906	2, 48, F=1.94, *P*=0.1549

[1] Units of titer is virus copy number per ng total DNA.
[2] Num DF, Den DF, F Value, Pr>F. Not significant ($P>0.05$) or significant at $P<0.05(^{*})$, $0.01(^{**})$ or $0.001(^{***})$. Significance values are listed by column.

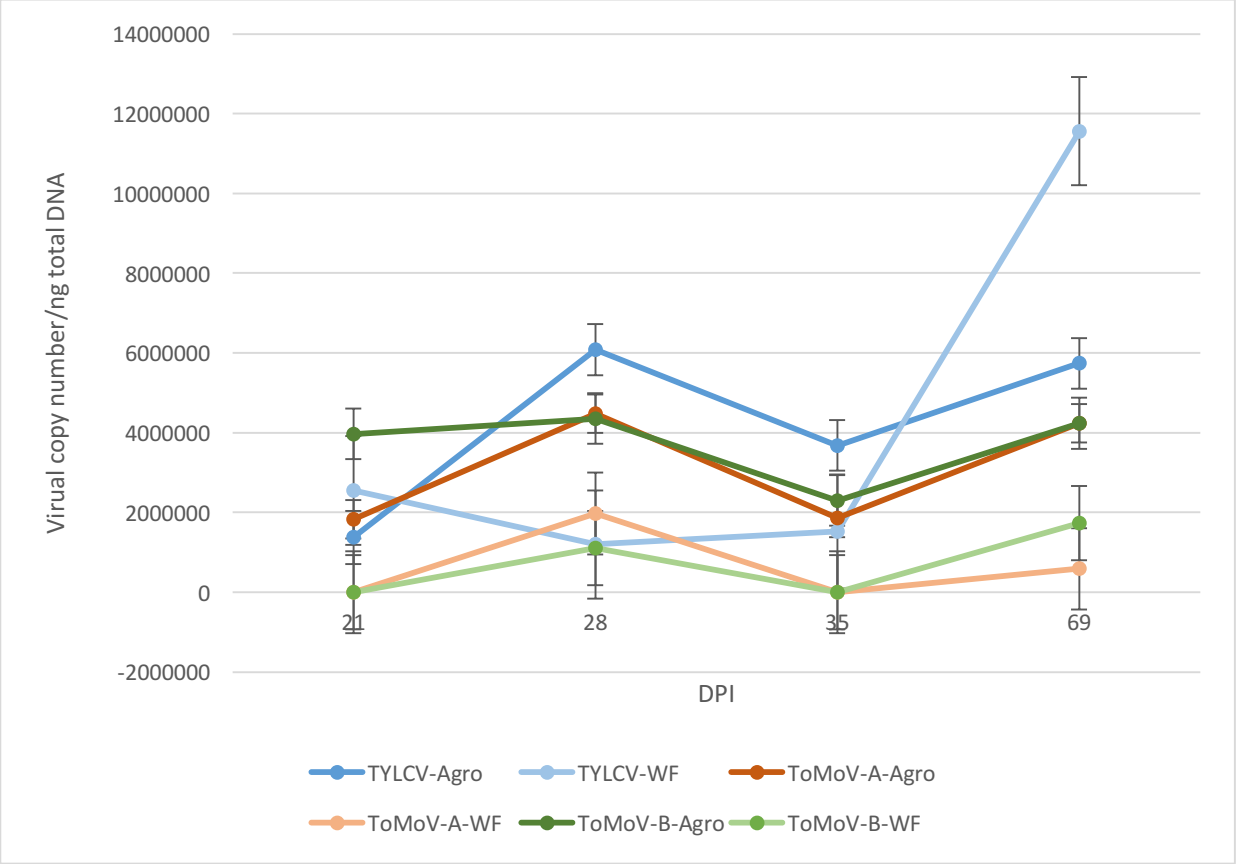

Figure 3.1. The mean (standard error) of virus titer of *Tomato yellow leaf curl virus* (TYLCV), *Tomato mottle virus* A component (ToMoV-A) and *Tomato mottle virus* B component (ToMoV-B) in Lanai tomato plants at 21, 28, 35 and 69 days post inoculation (dpi). Separate analyses were performed for whitefly transmitted and agroinoculated plants, and titers of TYLCV, ToMoV-A and ToMoV-B.

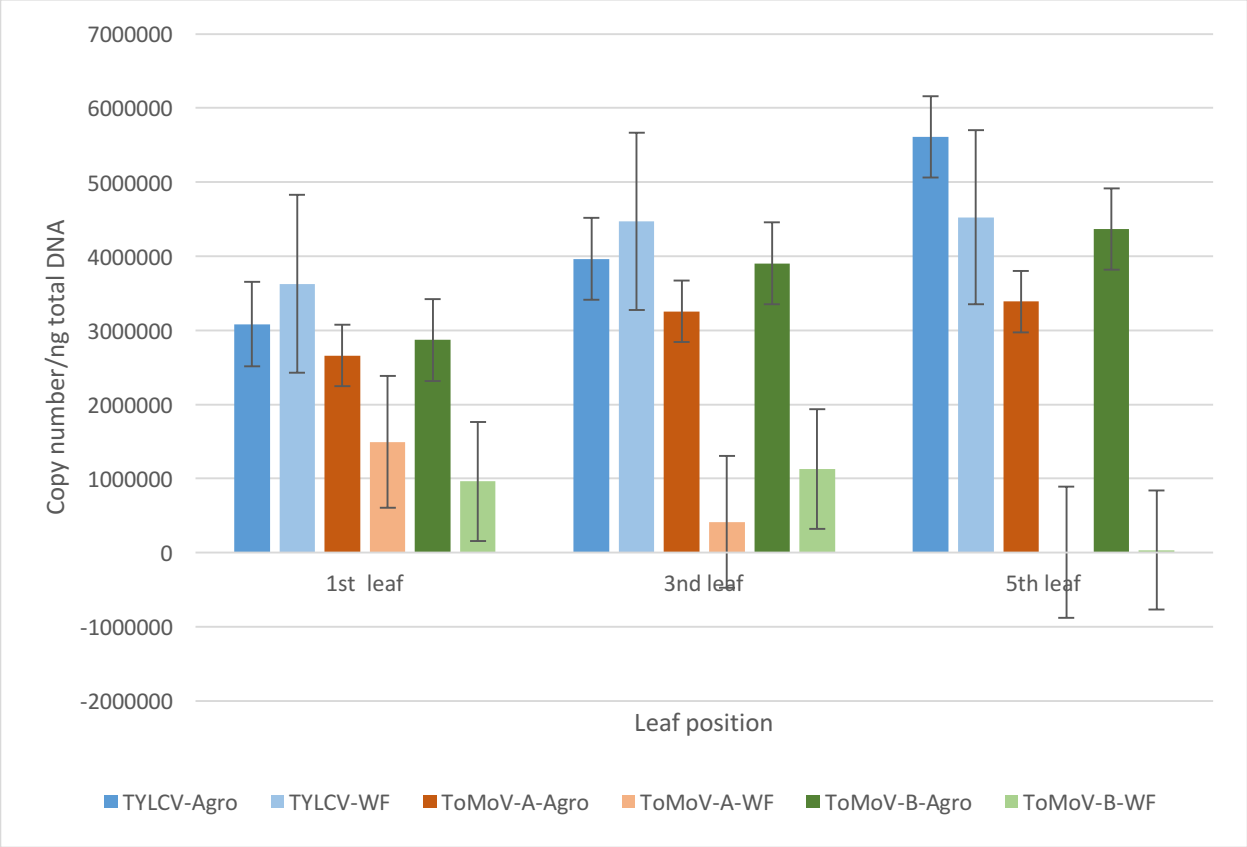

Figure 3.2. The mean (standard error) of virus titer of *Tomato yellow leaf curl virus* (TYLCV), *Tomato mottle virus* A component (ToMoV-A) and *Tomato mottle virus* B component (ToMoV-B) in the 1st, 3rd and 5th leaf in Lanai tomato plants. Separate analyses were performed for whitefly transmissmitted and agroinoculated plants, and titers of TYLCV, ToMoV-A and ToMoV-B.