Differential Transmission of Old and New World Begomoviruses by Middle East-Asia Minor 1 (MEAM1) and Mediterranean (MED) Cryptic Species of Bemisia tabaci

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Abstract: Middle East-Asia Minor 1 (MEAM1) and Mediterranean (MED) are two of the most invasive members of the sweetpotato whitefly, Bemisia tabaci, cryptic species complexes and are efficient vectors of begomoviruses. Bemisia tabaci MEAM1 is the predominant vector of begomoviruses in open-field vegetable crops in the southeastern United States. However, recently B. tabaci MED also has been detected in the landscape outside of greenhouses in Florida and Georgia. This study compared the transmission efficiency of one Old-World (OW) and two New-World (NW) begomoviruses prevalent in the southeastern United States, viz., tomato yellow leaf curl virus (TYLCV), cucurbit leaf crumple virus (CuLCrV), and sida golden mosaic virus (SiGMV) between B. tabaci MEAM1 and B. tabaci MED. Bemisia tabaci MEAM1 efficiently transmitted TYLCV, CuLCrV, or SiGMV, whereas B. tabaci MED only transmitted TYLCV. Percent acquisition and retention of OW TYLCV following a 72 h acquisition access period was significantly higher for B. tabaci MED than B. tabaci MEAM1. In contrast, B. tabaci MEAM1 acquired and retained significantly more NW bipartite begomoviruses, CuLCrV or SiGMV, than B. tabaci MED. Quantitative analysis (qPCR) of virus DNA in whitefly internal tissues revealed reduced accumulation of CuLCrV or SiGMV in B. tabaci MED than in B. tabaci MEAM1. Fluorescent in situ hybridization (FISH) showed localization of CuLCrV or SiGMV in the midgut of B. tabaci MED and B. tabaci MEAM1. However, localization of CuLCrV or SiGMV was only observed in the primary salivary glands of B. tabaci MEAM1 and not B. tabaci MED. TYLCV localization was observed in all internal tissues of B. tabaci MEAM1 and B. tabaci MED. Overall, results demonstrate that both B. tabaci MEAM1 and B. tabaci MED are efficient vectors of OW TYLCV. However, for the NW begomoviruses, CuLCrV and SiGMV, B. tabaci MEAM1 seems to be a better vector.

Keywords: cucurbit leaf crumple virus; midgut; primary salivary glands; sida golden mosaic virus; tomato yellow leaf curl virus

1. Introduction

Inadvertent introduction of a new species into geographical areas can cause large-scale ecological and economic consequences [1,2]. Insects form the biggest part of all non-native invasive animal species on the planet [3]. Introduced insects can interact with and competitively displace closely related indigenous species, and by serving as vectors, they can significantly alter the dynamics of dormant native or introduced pathogens [4–9].
In 2004, *Bemisia tabaci* Gennadius Mediterranean (MED, formerly known as Q biotype), was first detected in the United States in Arizona [10]. *Bemisia tabaci* MED is now present in greenhouses in 23 states in the United States [10,11]. For the first time, in 2016, *B. tabaci* MED was detected outside of the greenhouse in Florida, and more recently it was documented in snap bean fields located in proximity to urban landscapes in Georgia [12,13]. Consequently, the probability of *B. tabaci* MED moving into open crop production systems has increased in the southeastern United States.

*Bemisia tabaci* Middle East-Asia Minor 1 (MEAM1, formerly known as B biotype) and *B. tabaci* MED are members of the cryptic species complex [14–20]. In 1980, the introduction of the invasive *B. tabaci* MEAM1 in the southern United States resulted in significant changes in whitefly population dynamics. Unprecedented outbreaks of *B. tabaci* MEAM1 were observed in field-grown row and vegetable crops [21–25]. Due to greater polyphagy and stronger ability to adapt to different environmental conditions, *B. tabaci* MEAM1 quickly displaced the native *B. tabaci* A biotype [26,27]. A few years after the introduction of *B. tabaci* MEAM1 into the southern United States, epidemics of previously unreported whitefly-transmitted viruses were documented in Cucurbitaceae and Solanaceae crops [26,28,29]. Similar patterns of whitefly infestation and whitefly-transmitted virus epidemics have been observed in important vegetable crops in China and Brazil upon invasion and establishment of *B. tabaci* MED and *B. tabaci* MEAM1, respectively [30–35]. Likewise, detection of *B. tabaci* MED in the southeastern United States in the landscape/crop fields has raised serious concerns about the transmission dynamics involving prevalent and/or new viruses, especially begomoviruses as they account for 90% of the whitefly-transmitted viruses [36]. *Bemisia tabaci* MED has been shown to have a higher propensity to develop resistance to commonly used insecticides in whitefly management programs in vegetable crops [12,37,38]. Under high insecticide pressure, *B. tabaci* MED can outcompete, *B. tabaci* MEAM1 [39]. In China, Japan, and South Korea, *B. tabaci* MED has begun displacing *B. tabaci* MEAM1 as the predominant invasive *B. tabaci* cryptic species [40–43]. Studies from China have reported that tomato yellow leaf curl virus (TYLCV) is transmitted more efficiently by *B. tabaci* MED than *B. tabaci* MEAM1, and the recent epidemics of TYLCV in China have been associated with outbreaks of *B. tabaci* MED [44,45].

Begomoviruses are transmitted in a persistent circulative manner by whiteflies in the *B. tabaci* cryptic species complex [46,47]. For successful inoculation upon acquisition, begomoviruses must overcome the midgut (MG) and the primary salivary glands (PSG) barriers [48,49]. Capsid protein is the only known virus-encoded protein that is reported to mediate begomovirus interactions within whiteflies [50]. The amino acids between two conserved regions in the capsid protein, viz., GCEGPCKVQS and LYMACTHASN, seem to influence begomovirus movement in *B. tabaci* [51–53]. Begomoviruses with high capsid protein sequence similarity may share similar transmission patterns and could be cryptic-species-specific [52]. For instance, viruses with high capsid protein similarities, viz., tobacco curly shoot virus (TbCSV) and the cotton leaf curl Multan virus (CLCuMuV), were more efficiently transmitted by *B. tabaci* Asia II 1 than by *B. tabaci* MEAM1. This was presumably because both viruses were able to efficiently cross the midgut barrier in *B. tabaci* Asia II 1 than in *B. tabaci* MEAM1 [52]. Other than the virus capsid protein, many vector factors might also be involved in begomovirus interactions within whiteflies [53–55]. However, those remain poorly understood. Depending on the interactions between virus capsid protein, endosymbionts and/or vector factors, different *B. tabaci* cryptic species have been documented to transmit viruses with varying efficiencies. For instance, inefficient transmission of TYLCV by *B. tabaci* MED in comparison with *B. tabaci* MEAM1 is reported to be dependent on the presence of an endosymbiotic bacterium (*Hamiltonella*) in *B. tabaci* MEAM1 and its absence in *B. tabaci* MED populations [36].

Tomato (*S. lycopersicum*), squash (*Cucurbita pepo* L.), and common (snap) bean (*Phaseolus vulgaris* L.) are important summer crops in the southeastern United States. Tomato-infecting tomato yellow leaf curl virus (TYLCV), squash- and snap-bean-infecting cucurbit leaf crumple virus (CuLCrV), and snap-bean-infecting sida golden mosaic virus (SiGMV)
are important whitefly-transmitted begomoviruses in Georgia and Florida. TYLCV is a monopartite Old-World (OW) begomovirus that was introduced into the southern United States in the 1990s [57]. CuLCrV and SiGMV are bipartite New-World (NW) begomoviruses that are endemic to the Americas [58,59]. Currently, under field conditions, TYLCV, CuLCrV, and SiGMV are transmitted by B. tabaci MEAM1 [13]. To assess the possible impact of B. tabaci MED on virus epidemics, the current study compared the transmission of TYLCV, CuLCrV, or SiGMV by B. tabaci MED and B. tabaci MEAM1. This study also assessed the acquisition and retention (for 28 days) of TYLCV, CuLCrV, or SiGMV by B. tabaci MED and B. tabaci MEAM1. Furthermore, this study conducted quantitative (qPCR) and qualitative (fluorescence in situ hybridization, FISH) investigations to quantitate and localize TYLCV, CuLCrV, or SiGMV within B. tabaci MED and B. tabaci MEAM1 internal tissues. Lastly, based on the earlier studies that reported begomovirus transmission using B. tabaci MED, a capsid protein alignment was made and examined with the goal of identifying differences in the capsid proteins of B. tabaci MED transmissible and non-transmissible begomovirus species.

2. Materials and Methods

2.1. Plants, Virus Inoculum Sources, and B. tabaci

Tomato (S. lycopersicum L. cv. Florida 47, Harris Moran Seeds Company, Davis, CA, USA), yellow summer squash (C. pepo L. cv Gold Star F1 hybrid, Syngenta®, Willimington, DE, USA), and prickly sida (Sida spinosa L., from field-collected plants) seeds were planted in 1 L plastic pots using Grower Mix (Asb Greenworld, West Point, VA, USA). Plants were maintained in a greenhouse (25–30 °C, 14 h L:10 h D) in insect-proof cages (Megaview Science Co., Taichung, Taiwan) with 5 plants per cage. Plants were fertilized every alternate week with slow-release granular fertilizer, Osmocote (Scotts Miracle-Gro products, Inc., Marysville, OH, USA) as per label recommendations. The TYLCV isolate was collected from a commercial tomato farm located in Montezuma (Macon County, GA, USA) in 2009 and maintained since on TYLCV-susceptible tomato variety Florida 47 through repeated inoculations of three- to four-week-old tomato plants using viruliferous B. tabaci MEAM1. The CuLCrV isolate used in this study was collected from infected yellow summer squash plants from Tifton, GA, in 2016 and maintained since on susceptible yellow summer squash plants through repeated inoculations of two- to three-week-old squash plants using viruliferous B. tabaci MEAM1. The SiGMV isolate was collected from infected prickly sida plants growing amidst the natural vegetation at the UGA Horticulture Hill Farm in Tifton (Tift County, GA, USA) in 2018 and since maintained on prickly sida plants through repeated inoculations of four- to six-week-old prickly sida seedlings using viruliferous B. tabaci MEAM1 [59].

Bemisia tabaci MEAM1 (Genbank accession number: MN970031) used in the present study was collected in 2009 from a cotton farm in Tifton Georgia and since reared on cotton plants in whitefly-proof cages in the greenhouse at above-stated conditions. Bemisia tabaci MED (GenBank accession number: MZ469725) was collected from poinsettia plants from a nursery located in North Georgia, USA by Dr. Ronald Oetting, UGA. Since 2017, a B. tabaci MED colony has been maintained on cotton plants in whitefly-proof cages in the greenhouse at the above-stated conditions. Every alternate month, five to ten individuals from each colony were tested for the purity of the colonies by partially sequencing the mtCOI gene using universal COI primers C1- J-2195 and L2-N-3014 [60,61].

2.2. Bemisia tabaci Fitness on Tomato, Squash, and Prickly Sida Plants

To assess host suitability, life-history parameters (fecundity, egg–adult survival, developmental time) of B. tabaci MEAM1 and B. tabaci MED on three plant species (tomato, squash, and prickly sida) were measured. Three two-true leaf stage plants were placed in insect-proof cages in the greenhouse and maintained at the above-stated conditions. Using a clip cage (36.5 mm × 25.4 mm × 9.5 mm), two pairs of recently emerged (up to 48 h old) B. tabaci MEAM1 or B. tabaci MED adults were attached to the abaxial side of a leaf on
each plant and provided with a 48 h oviposition access period (OAP). Post OAP, whitefly adults were removed, and total number of eggs laid were recorded under the dissecting microscope at 10× magnification (MEIJI TECHNO, Santa Clara, CA, USA). Using a 0.1 mm diameter micro pin mounted on a chopstick, the number of eggs per cage were adjusted to five. Cages were monitored daily at ~10 AM until adult emergence. Egg to adult survival and developmental time from egg to adult of B. tabaci were recorded. The experiment was conducted three times (n = 45 for each whitefly and plant combination). To assess fecundity, a pair of B. tabaci MEAM1 or B. tabaci MED whitefly adults (up to 24 h old) that developed on tomato, squash, and prickly sida plants were clip caged to two-to-three true leaf stage tomato, squash, and prickly sida plants. Whitefly adults were transferred to new clip cages every fifth day for the next 15 days. Egg-bearing leaves were excised and numbers of eggs were enumerated under a dissecting microscope (10×). Each treatment had 10 replications (10 clip cages), and the experiment was conducted three times (n = 30 for each treatment).

2.3. Virus Detection

TYLCV infection status of tomato plants and viruliferous whiteflies was determined through endpoint PCR performed using the primers and conditions described earlier by Legarrea et al. (2015) [62]. Briefly, 5 µL GoTaq Green Mastermix buffer (2×) (Promega, Madison, WI, USA) was combined with primers C2-1159 and C2-1853 (final concentration 0.5 µM) (Table 1), 20 ng DNA, and nuclease-free distilled water for a final volume of 10 µL. PCR was performed using a T-100 thermocycler. An initial denaturation step (2 min at 95 °C) was followed by 40 cycles of 95 °C for 1 min, 58 °C for 1 min, and 72 °C for 1 min, and a final extension of 72 °C for 10 min.

CuLCrV or SiGMV infection status in infected plants and viruliferous whiteflies was determined through endpoint PCR using the primers and conditions described earlier by Gautam et al. (2020) and Gautam (2019), respectively [59,63]. Briefly, for CuLCrV, 5 µL GoTaq Green Mastermix buffer (2×) was combined with primers 3FAC3 and 5RAC1 (final concentration 0.5 µM) (Table 1), 20 ng DNA, and nuclease-free distilled water for a final volume of 10 µL. PCR was performed using a T-100 thermocycler. An initial denaturation step (2 min at 95 °C) was followed by 40 cycles of 95 °C for 1 min, 54 °C for 1 min, and 72 °C for 1 min, and a final extension of 72 °C for 10 min. The presence of SiGMV was tested using primers SiGMVF and SiGMVR (final concentration 0.5 µM) (Table 1). The primers were combined with 20 ng DNA and nuclease-free distilled water for a final volume of 10 µL. PCR was performed using a T-100 thermocycler. An initial denaturation step (2 min at 95°C) was followed by 40 cycles of 95°C for 1 min, 58°C for 1 min, and 72°C for 1 min, and a final extension of 72°C for 10 min. PCR products were run on 1% agarose gel containing GelRed at 0.5 µg/mL (Biotium, Fremont, CA, USA) and observed under UV light.

| Virus     | GenBank Accession Numbers | Name       | Sequence (5′–3′) | Product Size (bp) |
|-----------|---------------------------|------------|-----------------|-------------------|
| TYLCV     | KY971372                  | TYLC-C2-For | GCAGTGATGAGTTCCCCTGT | 102               |
|           |                           | TYLC-C2-Rev | CCAATAAGGCCTACGCTG |                   |
|           |                           | C2-1159    | CTAGATCCACTGCTCTGATA | 694               |
|           |                           | C2-1853    | TCATTGATGACCTAGCAAAG |                   |
| CuLCrV    | AF224760                  | CuLCrV-QF  | CTCGAAGTTTCTGCCCTCT | 110               |
|           |                           | CuLCrV-QR  | CCGATAGATCTGCGGCTGTC |                   |
|           |                           | 3FAC3      | TTTATATCATGATTTGCGAGTACA | 525               |
|           |                           | 5RAC1      | AAAATGAAAGCCTAAGAGAGTGGA |               |
| SiGMV     | MK387701                  | SiGMV-QF   | CTCGAAGTTTACCCGAAC | 114               |
|           |                           | SiGMV-QR   | CGGATAGATCTGCGGCTGTCCT |                   |
|           |                           | SiGMVVF    | TTCTCTCGTGACGATAGTG |                   |
|           |                           | SiGMVR     | ACTGGCAGCTCTGTGTA |                   |

CuLCrV or SiGMV infection status in infected plants and viruliferous whiteflies was determined through endpoint PCR using the primers and conditions described earlier by Gautam et al. (2020) and Gautam (2019), respectively [59,63].
2.4. Virus Quantitation

For virus quantitation in samples of infected plants or viruliferous whiteflies, extracted total genomic DNA (plants 10 ng or whiteflies 20 ng) was subjected to qPCR. TYLCV accumulation in samples was determined using conditions described earlier by Legarrea et al. (2015) [62]. Briefly, primers TYLC-C2-For and TYLC-C2-Rev (final concentration 0.25 µM) were combined with 6.25 µL GoTaq qPCR Master Mix (2×) (Promega, Madison, WI, USA), DNA (plants 10 ng or whiteflies 20 ng), and nuclease-free water for a final volume of 12.5 µL. Quantitative PCR was performed in Mastercycler ep realplex4 (Eppendorf, Hauppauge, NY, USA) with the following conditions: an initial denaturation step (2 min at 95 °C) followed by 40 cycles of denaturation (15 s at 95 °C) and a combined step of annealing and extension at 65 °C for 20 s. Finally, melting curve analysis was conducted to evaluate the specificity of the fluorescence signal. Absolute virus copy numbers were estimated using the procedure described by Legarrea et al. (2015) [62].

CuLCrV or SiGMV accumulation in infected plants or viruliferous whiteflies was determined using the conditions described earlier by Gautam et al. (2020) and Gautam (2019), respectively [59,63]. Primers CuLCrV-QF and CuLCrV-QR were used for CuLCrV quantitation, and SiGMV-QF and SiGMV-QR were used for SiGMV quantitation (Table 1). Primers, DNA, master mix, and water were used in the same proportions as described above for TYLCV. For CuLCrV, cycling parameters were as follows: 95 °C for 2 min followed by 40 cycles of 95 °C for 1 min, 63 °C for 15 s, and 72 °C for 20 s. Cycling parameters for SiGMV included an initial denaturation step (2 min at 95 °C) followed by 40 cycles of 95 °C for 15 s, 63 °C for 10 s, and 72 °C for 20 s. Melting curve analysis was performed to evaluate the specificity of the fluorescence signal, and absolute virus copy numbers were estimated using the procedure described earlier by Legarrea et al. (2015) [62].

2.5. TYLCV, CuLCrV, or SiGMV Transmission by B. tabaci MEAM1 and B. tabaci MED

Virus inoculum sources were maintained in susceptible plants through viruliferous B. tabaci MEAM1-mediated transmission. Therefore, to avoid contamination of B. tabaci MEAM1 in B. tabaci MED transmission assays, insect-free virus inoculum sources were generated. To generate insect-free TYLCV-infected tomato, CuLCrV-infected squash, or SiGMV-infected prickly sida plants, viruliferous B. tabaci MEAM1 were obtained by providing whiteflies a 72 h acquisition access period (AAP) on infected plants (TYLCV-infected tomato, CuLCrV-infected squash, or SiGMV-infected prickly sida). Using clip cages, viruliferous B. tabaci MEAM1 (~100/plant) was attached to the first true leaves of a non-infected two-true-leaf-stage plants (tomato, squash, or prickly sida). Individual plants with clip cages were placed in insect-proof cages under the conditions described above. One week after removing B. tabaci from inoculated plants, all leaves from the inoculated plants were excised, and plants without leaves were transferred to new insect-free cages. Three weeks post leaf excision, infection in the emerging symptomatic foliage was confirmed through endpoint PCR (described above). To further ensure that no whiteflies were present on the plants, the foliage of insect-free virus-infected plants was carefully examined for whitefly adults, nymphs and/or eggs using a hand lens under a fluorescent white light lamp.

Viruliferous whiteflies used for transmission studies were obtained by releasing 1500 B. tabaci MEAM1 or B. tabaci MED for 72 h (AAP) on the insect-free infected plants (TYLCV-infected tomato, CuLCrV-infected squash, or SiGMV-infected prickly sida). Following the AAP, 20 whiteflies were randomly collected, and total genomic DNA from individual whiteflies was extracted in 50 µL InstaGene Matrix containing six percent Chelex resin (Bio-Rad, Hercules, CA). Extracted total genomic DNA (20 ng) was subjected to endpoint PCR using the primers and conditions described above.

Batches (10 × 3) of viruliferous B. tabaci MEAM1 or B. tabaci MED for which at least 60% of individuals tested positive via PCR analysis were used for transmission studies (B. tabaci MEAM1 (TYLCV: 90–100%; CuLCrV: 70–90%; SiGMV: 80–90%); B. tabaci MED (TYLCV: 95–100%; CuLCrV: 60–80%; SiGMV: 65–85%)). Using a clip cage, viruliferous whiteflies (100 adults/plant) were attached to the first true leaf of a two-leaf stage non-
infected plant (tomato, squash, or prickly sida plants). Plants were then held in insect-proof
cages in the greenhouse under conditions described above. After a 5-day inoculation
access period (IAP), plants were treated with the insecticide, Admire pro® (imidacloprid,
Bayer CroPscience, Research Triangle Park RTP, NC) as per label recommendations via soil
drenching. Four weeks later, the topmost leaf sample (100 mg) was excised and surface-
sterilized using a six-step surface sterilization protocol [64,65]. Using a squeeze bottle,
collected leaf samples were washed in sterile distilled water, followed by 30 s rinse in
1% bleach, followed by a 30 s wash in 70% ethanol, and three more rinses with sterile
distilled water. Water from the last rinsate was collected in a sterile 1.5 mL centrifuge
tube. The surface-sterilized leaf tissues were used for DNA extraction. Total genomic DNA
was extracted with the GeneJET Plant Genomic Purification Kit (ThermoFisher Scientific,
Waltham, MA, USA). Virus infection status in plants was determined through end-point
PCR using conditions and programs described above, and the virus was quantitated in
infected plant samples using the qPCR protocols described above. Each treatment had 10
replications and the experiment was conducted three times (n = 30). Surface sterilization
and PCR analysis of water from the last rinsate eliminated potential false positives that
may have arisen due to residual virus and/or viral DNA-laden honeydew secreted by
viruliferous whiteflies on the samples. Prior to the transmission studies, the efficiency
of surface sterilization in removing the residual honeydew sticking on the samples was
confirmed with TYLCV-infected tomato plants and B.tabaci MEAM1 following TYLCV
acquisition (Supplementary Material, Table S1).

2.6. Retention of TYLCV, CuLCrV, or SiGMV by B. tabaci MEAM1 and B. tabaci MED

_Bemisia tabaci_ MEAM1 or _B. tabaci_ MED adults were allowed to feed on insect-free non-
infected or virus-infected plants for 72 h. Whiteflies were then transferred to four-week-old
cotton plants. Cotton is a non-host for all three viruses (Supplementary Material Table S2).
Twenty whitely adult were randomly collected from the cotton plants at five separate
time intervals (72 h, 7 d, 14 d, 21 d, and 28 d). After surface sterilization, total genomic
DNA from individual whiteflies was extracted using the protocol described above. TYLCV,
CuLCrV, or SiGMV presence and accumulation at each interval were estimated using
endpoint PCR and qPCR, respectively. Each treatment had 20 whiteflies. The experiment
was conducted three times (n = 60 for each treatment).

2.7. TYLCV, CuLCrV, or SiGMV Localization and Accumulation in B. tabaci MEAM1 and
B. tabaci MED Tissues

_Bemisia tabaci_ MEAM1 and _B. tabaci_ MED adults were transferred onto insect-
free infected or non-infected plants (TYLCV-infected or non-infected tomato, CuLCrV-
infected or non-infected squash, or SiGMV-infected or non-infected prickly sida). Whiteflies
were allowed to feed on infected or non-infected plants for 72 h, after which whiteflies
were transferred to four-week-old cotton plants. After 72 h, twenty individual whiteflies
from cotton were tested for the presence of the virus. Feeding on virus non-host cotton
ensured removal of free-floating virus particles from whiteflies midgut lumen. Using
an aspirator, viruliferous or non-viruliferous whiteflies were collected in 10 mL plastic
vials and chilled on ice. Individual adult whiteflies were collected with the help of a fine
paint brush, then using fine-tip forceps (Dumont Tweezer, Style 5, Electron Microscopy
Science, Hatfield, PA, USA) and a 0.1 mm diameter micro pin (Roboz Surgical Instrument,
Gaithersburg, MD, USA) mounted on a chopstick, the midgut (MG) and primary salivary
glands (PSG) were dissected on a glass slide with a single concave depression cavity in
saline buffer (200 µL). The fluorescence in situ hybridization (FISH) procedure followed
the protocols described by Kliot et al. 2014 [66]. Briefly, dissected organs were fixed
using Carnoy’s fixative (VWR, Monroeville, PA, USA). Following fixation, organs were
washed with hybridization buffer (20 mM Tris-HCl pH 8.0, 0.9 M NaCl, 0.01% (w/v)
sodium dodecyl sulfate, and 30% (v/v) formamide). After washing, specimens were
hybridized overnight at 4°C in 500 µL hybridization buffer supplemented with 10 pmol
of Cy3-labeled fluorescent oligonucleotide probes. After 12 h, organs were transferred using a custom-made eyelash brush into 30 µL of hybridization buffer containing DAPI (0.1 mg/mL in 1 x PBS, Gene TEX, CA) on a new glass slide. After 5 min, organs were gently covered with a glass cover slip, sealed with Permount (Fisher Scientific, Waltham, MA, USA), and observed under an Olympus BX 60 fluorescence microscope (Olympus Corporation of the Americas, Center Valley, PA). TYLCV was localized using the TYLCV-probe [Cy3 5′-GGAACATCAGGGCTTCGATA-3′] reported earlier by Kliot et al. (2014) [66]. The DNA probes, namely CuLcrV-Probe: [Cy3 5′-GCCGAAGCGGATGCCCAT-3′] and SiGMV-Probe: [Cy3 5′-CAAGGCCCTCTGAATGGGTAA-3′] specific to the DNA-A of CuLcrV and SiGMV were designed using primer3 software [67].

For virus quantitation, MG, hemolymph, and PSG from 25 whiteflies were obtained for each treatment. MG and PSG were dissected using the procedure described above, and hemolymph was collected using the procedure described earlier by He et al. [68]. After dissection, MG, hemolymph, and PSG collected from five whiteflies (belonging to same treatment) were pooled and treated as one experimental replicate. A total of five experimental replicates were generated per treatment. Total DNA from samples was extracted using the procedure described above for individual whiteflies. DNA was subjected to qPCR analysis using the procedure described above. Each treatment had five replications. The experiment was conducted two times (n = 10).

2.8. Comparison of Capsid Protein Sequences of B. tabaci MED Transmissible and Non-Transmissible Begomoviruses

A total of 17 begomoviruses for which B. tabaci MED-mediated transmission studies have been conducted prior to the current study were identified (Table 2). Capsid protein sequences of the 17 viruses were aligned with the capsid protein sequences of TYLCV, CuLcrV, and SiGMV isolates used in the current study. The sequences were aligned in Geneious Prime, and aligned sequences were visualized in the Espript 3.0 [69,70].

| Sr. No. | Virus                              | Transmission Efficiency (No. of Viruliferous Whitefly Used Plant | Accession Number | Type a | Reference |
|--------|------------------------------------|---------------------------------------------------------------|------------------|--------|-----------|
| 1      | Cucurbit leaf crumple virus (CuLCrV) | 0% (100)                                                       | AF256200         | NW (bipartite) | Current study |
| 2      | Sida golden mosaic virus (SiGMV)    | 0% (100)                                                       | MK387701         | NW (bipartite) | Current study |
| 3      | Sida golden yellow vein virus (SiGYVV) | 0% (50)                                                      | HQ822123         | NW (bipartite) | [71]       |
| 4      | Bean golden mosaic virus (BGMV)     | 100% (10)                                                      | NC_004042        | NW (bipartite) | [72]       |
| 5      | Tomato severe rugose virus (ToSRV) | 10% (15)                                                       | MG837738         | NW (bipartite) | [73]       |
| 6      | Tomato yellow spot virus (ToYSV)    | 0% (15)                                                        | DQ336350         | NW (bipartite) | [73]       |
| 7      | Tomato leaf deformation virus (ToYLDV) | 0% (50)                                                      | NC_014510        | NW (monopartite) | [71] |
| 8      | Tomato yellow leaf curl China virus (TYLCCNV) | 0% (10)                                                  | AJ319675         | OW (monopartite) | [53] |
| 9      | Tobacco curly shoot virus (TbCSV)   | 75% (5)                                                        | AJ420318         | OW (monopartite) | [74] |

Table 2. Bemisia tabaci MED transmissible and non-transmissible begomoviruses.
Table 2. Cont.

| Sr. No. | Virus                                                   | Transmission Efficiency (No. of Viruliferous Whitefly Used Plant) | Accession Number | Type ⁴ | Reference |
|---------|--------------------------------------------------------|-----------------------------------------------------------------|------------------|--------|-----------|
| 10      | Tomato yellow leaf curl virus Sardinia virus (TYLCSV-ES) | 40% (2)                                                          | Z25751           | OW (monopartite) | [75]      |
| 11      | Tomato yellow leaf curl virus Israel strain (TYLCV)     | 50% (2)                                                          | KY965923         | OW (monopartite) | [45]      |
| 12      | Cotton leaf curl Multan virus (CLCuMuV)                | 0% (10)                                                         | KP762786         | OW (monopartite) | [76]      |
| 13      | Papaya leaf curl China virus (PaLCuCNV)                | ~60% (10)                                                       | AM691554         | OW (monopartite) | [77]      |
| 14      | Tomato leaf curl Taiwan virus (ToLCTWV)               | 45% (5)                                                         | DQ866122         | OW (monopartite) | [78]      |
| 17      | Tomato leaf curl New Delhi virus (ToLCNDV)-ES          | 100% (25–30)                                                   | KF749224         | OW (bipartite)  | [79]      |
| 18      | Sri Lankan cassava mosaic virus (SLCMV)               | 63.3% (10)                                                       | MH891840         | OW (bipartite)  | [80]      |
| 19      | Tomato yellow leaf curl Thailand virus (TYLCTHV)       | 50% (5)                                                         | GU723742         | OW (bipartite)  | [78]      |
| 20      | Ramie mosaic virus (RaMoV)                            | ~100% (2)                                                        | KU522485         | OW (bipartite)  | [81]      |

⁴ NW = New World; OW = Old World.

2.9. Statistical Analyses

Data analyses were performed in R version 3.4.2 [82]. Data for egg–adult survival (dead vs. survived) were analyzed using a generalized mixed effect model in the lme4 package using glmer function and setting the family argument as binomial [83]. Within-model treatments were considered as the fixed effect and replications as the random effect. ANOVA was run using the function Anova in the package car [84]. Post hoc testing was performed using glht function with Tukey contrast in the multcomp package [85]. The median developmental time from egg to adult was analyzed by a non-parametric Wilcoxon rank-sum test (Mann–Whitney U test). Data on oviposition were analyzed using the linear mixed effect model (lmer function) in the Lme4 package with treatments as the fixed effect and replications as the random effect [83]. ANOVA was run using the function anova in the lmerTest package and post hoc testing was performed using the multcomp package [85,86]. Percent infection in plants (infected vs. non-infected) was evaluated using a generalized mixed-effect model in the lme4 package using the procedure described above [83]. One-way ANOVA and a repeated measures ANOVA were used to compare percent infection and virus retention in B. tabaci MEAM1 and B. tabaci MED at different time intervals. Percent infection and virus accumulation were analyzed using a generalized mixed-effect model and a linear-mixed effect model, respectively. Virus accumulation in whitefly adults and tissues was analyzed using a linear mixed-effect model. Statistical differences were considered significant at \( p < 0.05 \).

3. Results

3.1. Bemisia tabaci Performance on Tomato, Squash, or Prickly Sida Plants

Life history parameters between B. tabaci MEAM1 and B. tabaci MED developing on each host plant did not vary significantly (Table 3). Egg to adult survival, developmental
time, and fecundity on tomato, squash, or prickly sida plants were not significantly different between B. tabaci MEAM1 and B. tabaci MED (Table 3). Furthermore, no significant differences were observed in the fecundity of B. tabaci MEAM1 and B. tabaci MED on tomato, squash, or prickly sida plants (Table 3).

Table 3. Bemisia tabaci MEAM1 and B. tabaci MED life history parameters on tomato, squash, or prickly sida plants.

| Plant Type      | Whitefly Type | N  | Percent Egg Survival | Egg-Adult b | N  | Fecundity |
|-----------------|---------------|----|----------------------|-------------|----|-----------|
| Tomato          | MEAM1         | 45 | 80 ± 3.80            |             | 21 (18–29) | 30 | 72 ± 7.20 | F1,58 = 3.59 |
|                 | MED           | 45 | 73 ± 10.20           |             | 21 (17–30) | 30 | 82 ± 5.30 | p = 0.16 |
| Squash          | MEAM1         | 45 | 78 ± 8.00            |             | 21 (19–30) | 30 | 68 ± 13.50 | F1,58 = 1.09 |
|                 | MED           | 45 | 69 ± 9.70            |             | 22 (18–28) | 30 | 53 ± 5.30 | p = 0.41 |
| Prickly sida    | MEAM1         | 45 | 56 ± 4.40            |             | 23 (19–32) | 30 | 34 ± 9.20 | F1,58 = 4.76 |
|                 | MED           | 45 | 53 ± 10.20           |             | 24 (19–33) | 30 | 36 ± 2.90 | p = 0.09 |

a Number of eggs monitored to adulthood. b Median developmental time (days) from egg to adult with range in parentheses. c Number of B. tabaci pairs used for fecundity studies.

3.2. TYLCV, CuLCrV, or SiGMV Transmission and Quantitation

Percent transmission of TYLCV did not differ between MEAM1 and MED whiteflies ($\chi^2_{1,58} = 0; p = 1$). Furthermore, TYLCV accumulation did not differ between tomato plants inoculated by viruliferous B. tabaci MEAM1 or B. tabaci MED ($F_{1,28} = 2.15; p = 0.75$) (Figure 1A,B). However, CuLCrV transmission percentages by MEAM1 and MED whiteflies differed significantly ($\chi^2_{1,47} = 24.04; p < 0.001$). More than 80% of CuLCrV infection was observed in squash plants when inoculated with viruliferous B. tabaci MEAM1. In contrast, none of the squash plants inoculated with viruliferous B. tabaci MED were infected with CuLCrV (Figure 1A,C). Similarly, SiGMV percentage transmission differed significantly between B. tabaci MEAM1 and B. tabaci MED ($\chi^2_{1,56} = 29.14; p < 0.001$). More than 80% of SiGMV infection was observed in the prickly sida plants when inoculated with viruliferous B. tabaci MEAM1. In contrast, none of the prickly sida plants inoculated with viruliferous B. tabaci MED were infected with SiGMV (Figure 1A,D).

Figure 1. Percent virus infection and virus accumulation in plants inoculated with viruliferous B. tabaci MEAM1 and B. tabaci MED. (A), Bars with standard errors indicate percent TYLCV, CuLCrV, or SiGMV infection in tomato, squash, or prickly sida plants, respectively, inoculated by viruliferous
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MEAM1 or MED whiteflies. Viruliferous MEAM1 or MED whiteflies were obtained after 72 h AAP on TYLCV-infected tomato, CuLCrV-infected squash, or SiGMV-infected prickly sida plants. Viruliferous *B. tabaci* MEAM1 and *B. tabaci* MED adults were provided with a five-day inoculation access period (IAP) on non-infected two-true-leaf-stage tomato, squash, or prickly sida plants. Four weeks post-inoculation, infection status was confirmed via endpoint PCR. (B), Bars with standard errors represent average number of TYLCV copies per ng DNA in tomato plants. (C), Bars with standard errors represent average number of CuLCrV copies per ng DNA in squash plants. (D), Bars with standard errors represent average number of SiGMV copies per ng DNA in prickly sida plants. Y-axis on panels (B–D) is represented in logarithmic scale. Significant differences between means were separated with Tukey’s HSD posthoc test at $\alpha = 0.05$.

3.3. Retention of TYLCV, CuLCrV, or SiGMV by *B. tabaci* MEAM1 and *B. tabaci* MED

When viruliferous whitefly adults were transferred to virus non-host cotton, the percentages of viruliferous (TYLCV, CuLCrV, or SiGMV) whiteflies decreased over time as indicated by endpoint PCR (Figure 2A–C). At the end of the fourth week, approximately 73% of *B. tabaci* MEAM1 tested positive for TYLCV, 33% *B. tabaci* MEAM1 tested positive for CuLCrV, and 66% *B. tabaci* MEAM1 tested positive for SiGMV (Figure 2A–C). However, the percentages of viruliferous *B. tabaci* MED over five time intervals was virus-dependent. TYLCV incidence in *B. tabaci* MED did not decline gradually with time; at the end of the fourth week, about 90% of *B. tabaci* MED tested positive for TYLCV (Figure 2A). The percentage of *B. tabaci* MED that tested positive for CuLCrV dropped to 7% by the end of the fourth week (Figure 2B). Similarly, the percentage of *B. tabaci* MED that tested positive for SiGMV dropped to 27% by the end of the fourth week (Figure 2C).

Figure 2. Retention of TYLCV, CuLCrV, or SiGMV by *B. tabaci* MEAM1 and *B. tabaci* MED. Values are means ± SE. Means with different letters are significantly different as separated with Tukey’s HSD posthoc test at $\alpha = 0.05$. 

3.3. Retention of TYLCV, CuLCrV, or SiGMV by *B. tabaci* MEAM1 and *B. tabaci* MED

When viruliferous whitefly adults were transferred to virus non-host cotton, the percentages of viruliferous (TYLCV, CuLCrV, or SiGMV) whiteflies decreased over time as indicated by endpoint PCR (Figure 2A–C). At the end of the fourth week, approximately 73% of *B. tabaci* MEAM1 tested positive for TYLCV, 33% *B. tabaci* MEAM1 tested positive for CuLCrV, and 66% *B. tabaci* MEAM1 tested positive for SiGMV (Figure 2A–C). However, the percentages of viruliferous *B. tabaci* MED over five time intervals was virus-dependent. TYLCV incidence in *B. tabaci* MED did not decline gradually with time; at the end of the fourth week, about 90% of *B. tabaci* MED tested positive for TYLCV (Figure 2A). The percentage of *B. tabaci* MED that tested positive for CuLCrV dropped to 7% by the end of the fourth week (Figure 2B). Similarly, the percentage of *B. tabaci* MED that tested positive for SiGMV dropped to 27% by the end of the fourth week (Figure 2C).
post hoc test at $\alpha = 0.05$. Percent infection in \textit{B. tabaci} adults (MEAM1 and MED) collected from virus non-host cotton plants at five time intervals (72 h, 7 d, 14 d, 21 d, and 28 d) after a 72 h AAP on (A), TYLCV-infected tomato plants, (B), CuLCrV-infected squash plants, or (C), SiGMV-infected prickly sida plants. Virus accumulation in \textit{B. tabaci} adults (MEAM1 and MED) collected from cotton plants at five-time intervals (72 h, 7 d, 14 d, 21 d, and 28 d) after a 72 h AAP on (D), TYLCV-infected tomato plants, (E), CuLCrV-infected squash plants, or (F), SiGMV-infected prickly sida plants. Y-axis is shown in a logarithmic scale in panels (D–F).

When viruliferous whiteflies were transferred to virus non-host cotton, data from qPCR revealed that the amounts of TYLCV in \textit{B. tabaci} MEAM1 and \textit{B. tabaci} MED that had fed on TYLCV-infected tomato decreased gradually with time (Figure 2D). The amounts of CuLCrV or SiGMV also declined gradually in \textit{B. tabaci} MEAM1. However, in \textit{B. tabaci} MED, there was a sharp decline in the amount of CuLCrV or SiGMV after 72 h. \textit{Bemisia tabaci} MED retained more TYLCV than \textit{B. tabaci} MEAM1 at every time interval ($F_{9,528} = 8.69; p < 0.001$) (Figure 2D). However, $B. tabaci$ MEAM1 retained significantly more CuLCrV ($F_{9,228} = 10.58; p < 0.001$) (Figure 2E) and SiGMV ($F_{9,398} = 20.08; p < 0.001$) than $B. tabaci$ MED at every time interval (Figure 2F).

3.4. TYLCV, CuLCrV, or SiGMV Localization and Accumulation in \textit{B. tabaci} MEAM1 and \textit{B. tabaci} MED Tissues

Probes for TYLCV, CuLCrV, or SiGMV hybridized in viruliferous whitefly tissues. FISH revealed that TYLCV, CuLCrV, or SiGMV in \textit{B. tabaci} MEAM1 and \textit{B. tabaci} MED localized in the midgut (Figure 3). Most hybridization was localized within the filter chamber (Figure 3). There was some localization in the descending and ascending midgut as well. FISH signals for TYLCV were detected in $B. tabaci$ MEAM1 and $B. tabaci$ MED PSG (Figure 4A, D). However, CuLCrV or SiGMV localization was observed in the PSG of $B. tabaci$ MEAM1 alone and not in $B. tabaci$ MED (Figure 4B, C, E, F). In $B. tabaci$ MEAM1, TYLCV, CuLCrV, or SiGMV were primarily localized in the secretory section of the central region of PSG. No virus localization was detected in $B. tabaci$ MEAM1 or $B. tabaci$ MED tissues feeding on non-infected tomato, squash, or prickly sida plants.

TYLCV accumulation in $B. tabaci$ MED and $B. tabaci$ MEAM1 adults and tissues differed significantly ($F_{5,54} = 19.65; p < 0.001$) (Figure 5A). More TYLCV accumulated in $B. tabaci$ MED adults and in their MG in comparison with $B. tabaci$ MEAM1 adults and in their MG. However, TYLCV accumulation did not differ between hemolymph and PSG of $B. tabaci$ MED and $B. tabaci$ MEAM1. CuLCrV accumulation in $B. tabaci$ MED and $B. tabaci$ MEAM1 and in their tissues differed significantly ($F_{5,54} = 30.10; p < 0.001$) (Figure 5B). More CuLCrV accumulated in $B. tabaci$ MEAM1 adults, MG, hemolymph, and PSG than in $B. tabaci$ MED adults, MG, hemolymph and PSG. Similarly, SiGMV accumulation in $B. tabaci$ MED and $B. tabaci$ MEAM1 and their tissues differed significantly ($F_{5,54} = 104.55; p < 0.001$). More SiGMV accumulated in $B. tabaci$ MEAM1 adults and tissues than in $B. tabaci$ MED adults and tissues (Figure 5C).

3.5. Comparison of Capsid Protein Sequences of $B. tabaci$ MED Transmissible and Non-Transmissible Begomoviruses

An alignment of the capsid protein amino acid sequences of $B. tabaci$ MED transmissible and non-transmissible begomoviruses did not reveal consistent mutations of amino acids at any location (Figure 6). At amino acid residue 85 located between two conserved regions in the capsid protein, GCEGPCKVQS and LYMAGCTHASN, all $B. tabaci$ MED non-transmissible viruses except for tomato yellow leaf curl China virus (TYLCCV) had valine, whereas $B. tabaci$ MED-transmissible viruses had isoleucine (Figure 6).
Figure 3. TYLCV, CuLCrV, or SiGMV localization in the midgut dissected from *B. tabaci* adults (MEAM1 or MED) that fed on TYLCV-infected tomato, CuLCrV-infected squash, or SiGMV-infected prickly sida. FISH signals from Cy3-labeled virus-specific probe (red) and DAPI stained nuclei (blue) as seen from Olympus BX 60 fluorescence microscope at 200× magnification using Cy3 and DAPI filter cubes. FC: filter chamber; DM: descending midgut; AM: ascending midgut; CA: caeca; and HG: hindgut. Midgut of *B. tabaci* MEAM1 following (A), TYLCV acquisition, (B), CuLCrV acquisition and (C), SiGMV acquisition. Midgut of *B. tabaci* MED following (D), TYLCV acquisition, (E), CuLCrV acquisition, and (F), SiGMV acquisition.

Figure 4. TYLCV, CuLCrV, or SiGMV localization in the primary salivary glands (PSG) dissected from *B. tabaci* adults (MEAM1 and MED) that fed on TYLCV-infected tomato, CuLCrV-infected
squash, or SiGMV-infected prickly sida. FISH signals from Cy3-labeled virus-specific probe (red) and DAPI stained nuclei (blue) as seen from Olympus BX 60 fluorescence microscope at 400× magnification using Cy3 and DAPI specific filter cubes. DS—ductal section of the central region; EC—end cap; SS—secretory section of the central region. PSG of *B. tabaci* MEAM1 following (A), TYLCV, (B), CuLCrV, or (C), SiGMV acquisition. PSG of *B. tabaci* MED following (D), TYLCV, (E), CuLCrV, or (F), SiGMV acquisition.

**Figure 5.** Virus accumulation in *B. tabaci* adults and tissues. Bars with standard errors represent mean virus copy numbers per ng of total DNA within whole adults, midguts (MG), hemolymph (HM), and primary salivary glands (PSG) of *B. tabaci* MEAM1 and *B. tabaci* MED adults following a 72 h AAP on (A) TYLCV-infected tomato plants, (B) CuLCrV-infected squash plants, or (C) SiGMV-infected prickly sida plants after a 72 h gut clearing. Y-axis is represented in a logarithmic scale and significant differences between means were analyzed using Tukey’s test at $\alpha = 0.05$. 
Figure 6. Comparison of capsid protein amino acid sequences of *B. tabaci* MED transmissible and non-transmissible begomoviruses. New-World non-transmissible (red); Old-World non-transmissible (magenta); New-World transmissible (green); and Old-World transmissible (black) begomoviruses. A consensus sequence was generated using MultAlin criteria: uppercase is identity lowercase is consensus level > 0.5, ! is I or V, $ is L or M, % is F or Y, and # is N, D, Q, E, B or Z. At amino acid residue 85 (highlighted in yellow), all *B. tabaci* MED-non-transmissible viruses except for tomato yellow leaf curl China virus (TYLCCV) had valine, whereas *B. tabaci* MED-transmissible viruses had isoleucine.
4. Discussion

*Bemisia tabaci* MED has been in the United States since 2004, but it was restricted primarily to greenhouse-grown ornamentals [11]. However, *B. tabaci* MED has been recently detected outside of greenhouses in Florida and Georgia [12,13]. Recent reports of *B. tabaci* MED outside of the greenhouse have raised serious concerns on the implications for begomovirus epidemics in important vegetable crops in the southeastern United States.

The current study compared the transmission efficiency of three economically important begomoviruses prevalent in the southeastern United States, viz., *tomato yellow leaf curl virus* (TYLCV), *cucurbit leaf crumple virus* (CuLCrV), and *sida golden mosaic virus* (SiGMV) between *B. tabaci* MEAM1 and *B. tabaci* MED. Both *B. tabaci* MED and *B. tabaci* MEAM1 were efficient vectors of the OW begomovirus, TYLCV. That was not the case for NW bipartite begomoviruses, CuLCrV and SiGMV. *Bemisia tabaci* MED was able to acquire and retain TYLCV, CuLCrV, or SiGMV after a 72 h AAP on TYLCV-infected tomato, CuLCrV-infected squash, or SiGMV-infected prickly sida plants, respectively. However, it was only able to inoculate TYLCV. In contrast, *B. tabaci* MEAM1 was able to acquire, retain, and inoculate non-infected tomato, squash, or prickly sida plants with TYLCV, CuLCrV, or SiGMV, respectively, after a 72 h AAP on the homologous infected hosts. Results from qPCR suggest that CuLCrV or SiGMV accumulated in the midgut of *B. tabaci* MED, but due to inefficient translocation from MG into hemolymph, CuLCrV or SiGMV failed to accumulate in the PSG, resulting in the inability to transmit CuLCrV or SiGMV. Taken together, results confirm that *B. tabaci* MED and *B. tabaci* MEAM1 differ in their ability to transmit NW bipartite begomoviruses, viz., CuLCrV and SiGMV.

A few other studies also have documented the differential transmission of begomoviruses by *B. tabaci* cryptic species. For instance, *B. tabaci* MEAM1 and *B. tabaci* MED differed in their ability to acquire and inoculate TYLCV isolate Sh2 reported from China [45]. Biotype A (New World 1) of *B. tabaci* has been reported as a better vector of chino del tomate virus than *B. tabaci* MEAM1 [87]. In the current study, *B. tabaci* MED accumulated and retained a higher amount of TYLCV than *B. tabaci* MEAM1, although this did not translate into differences in transmission. Similar transmission efficiency between the two cryptic species (MEAM1 and MED) appear to be associated with the ability of whiteflies to acquire the virus particles well above the threshold that would otherwise limit virus inoculation. Upon transfer to virus non-host cotton, TYLCV accumulation declined gradually in *B. tabaci* MED (5–9%/day) and *B. tabaci* MEAM1 (8–14%/day). The results of this study are consistent with previous findings on the gradual decline of TYLCV in viruliferous whiteflies once transferred to a virus non-host plant [45]. However, the TYLCV decline observed in the current study is greater than the 1–2% per day as reported earlier [88,89]. Su et al. (2013) reported that the rate of TYLCV decline in viruliferous *B. tabaci* MED on virus non-host cotton was associated with the presence or absence of an endosymbiont, *Hamiltonella* [89]. Yet, in the current study, the symbionts’ genera reported to affect transmission of begomoviruses, viz., *Hamiltonella*, *Arsenophonus*, and *Rickettsia* were present in both *B. tabaci* MEAM1 and *B. tabaci* MED populations (Supplementary Material, Table S3). Therefore, higher decline rates of TYLCV in *B. tabaci* MEAM1 and *B. tabaci* MED observed in the current study might be the result of differences in other factors such as experimental conditions, virus isolates, and/or whitefly populations.

CuLCrV or SiGMV accumulation in *B. tabaci* MEAM1 and *B. tabaci* MED was lower than TYLCV. This might be because of differences in virus accumulation in the host plants. TYLCV accumulation in tomato plants, estimated as copies per ng DNA, was higher than CuLCrV or SiGMV accumulation in squash and prickly sida plants, respectively (Supplementary Material, Figure S1). This correlation between virus accumulation in the host and in the vector has also been reported in multiple whitefly-begomovirus pathosystems [62,63,90–92]. After transfer to a virus non-host cotton, within a week, a sharp decline (90%) in CuLCrV or SiGMV accumulation was observed for *B. tabaci* MED, whereas in *B. tabaci* MEAM1, CuLCrV or SiGMV declined gradually (6–10%/day). Czosnek et al. (2002) compared the retention of TYLCV between vector (*B. tabaci*) and non-vector (*Trialeu-
rodes vaporariorum) whiteflies. They reported that \textit{B. tabaci} was able to retain TYLCV for 15 days after a 4 d AAP on TYLCV-infected tomato, whereas TYLCV was undetectable in \textit{T. vaporariorum} within a few hours post transfer to a virus non-host [93]. In the current study, \textit{B. tabaci} MED acquired CuLCrV or SiGMV from infected plants after an AAP of 72 h and retained CuLCrV or SiGMV for much longer, albeit in lower amounts (>10-fold lower) than \textit{B. tabaci} MEAM1.

Some of the transmission differences between \textit{B. tabaci} cryptic species have been associated with differences in their feeding habits. For example, \textit{B. tabaci} MED has been documented to engage in prolonged and continuous feeding on TYLCV-infected plants than \textit{B. tabaci} MEAM1, translating into increased acquisition and subsequent inoculation of TYLCV by \textit{B. tabaci MED} [45]. In the current study, tomato, squash, and prickly sida plants were found to be equally susceptible to both \textit{B. tabaci} MED and \textit{B. tabaci} MEAM1. Therefore, it is possible that the differential transmission observed in this study might be the result of incompatible interactions between NW begomoviruses and \textit{B. tabaci} MED. To further examine the differential transmission of CuLCrV or SiGMV by \textit{B. tabaci MEAM1} and \textit{B. tabaci MED}, the current study, using qualitative (FISH) and quantitative (qPCR) techniques, traced the tropism of CuLCrV or SiGMV in \textit{B. tabaci MEAM1} and \textit{B. tabaci MED} adults. Both viruses were able to traverse the midgut barrier of \textit{B. tabaci MEAM1} and \textit{B. tabaci MED} adults. However, CuLCrV or SiGMV movement across the midgut of \textit{B. tabaci MED} was less efficient in comparison with \textit{B. tabaci MEAM1}. Lower-efficiency translocation across the \textit{B. tabaci MED} midgut resulted in reduced virus accumulation in the hemolymph and consequently in the PSG, leading to an inability to transmit CuLCrV or SiGMV. Similar results have been reported for cotton leaf curl Multan virus (CLCuMuV), where inefficient movement of CLCuMuV across the MG of \textit{B. tabaci MEAM1} and \textit{B. tabaci MED} adults in comparison with \textit{B. tabaci Asia II 1} resulted in reduced transmission of CLCuMuV in cotton [52]. None of the cotton plants inoculated by viruliferous \textit{B. tabaci MEAM1} and \textit{B. tabaci MED} were infected with CLCuMuV, whereas 52% of cotton plants inoculated by viruliferous \textit{B. tabaci Asia II 1} were infected with the CLCuMuV [52]. The reduced translocation of CuLCrV or SiGMV across the midgut and accumulation in salivary glands was substantiated via FISH and qPCR. In the current study, qPCR analysis revealed that CuLCrV or SiGMV accumulation levels in the PSG of \textit{B. tabaci MEAM1} was ~ 100-fold more than in the PSG of \textit{B. tabaci MED}. Additionally, FISH assay did not result in the localization of CuLCrV or SiGMV in the PSG of \textit{B. tabaci MED}. The non-detection of FISH signals corresponding to CuLCrV or SiGMV in the PSG of \textit{B. tabaci MED} might have been the consequence of reduced accumulation of CuLCrV or SiGMV in the PSG of \textit{B. tabaci MED} than \textit{B. tabaci MEAM1}. A previous study on TYLCV localization in \textit{B. tabaci MED} using qPCR and FISH also demonstrated that qPCR allowed more sensitive and specific detection of TYLCV in whitefly tissues than FISH [94].

Capsid protein is the only known begomovirus-encoded protein that affects transmission by whiteflies [50]. Capsid protein amino acids between the two conserved regions CEGPCKVQ5 and LYMCTHASN have been reported to play a crucial role in determining begomovirus transmissibility by whiteflies [51–53]. Single or a combination of changes in the amino acid residues between these conserved regions has been shown to have a profound impact on begomovirus transmissibility by a whitefly cryptic species [55,95,96]. For instance, a double mutation from glutamine to proline and glutamine to histidine at amino acid residues 129 and 134, respectively, in the capsid protein of tomato leaf curl Sardinia virus (TYLCV-Sar) resulted in the loss of \textit{B. tabaci} transmissibility [97]. Similarly, non-transmissible abutilon mosaic virus (AbMV) isolate was readily transmitted by the M biotype of \textit{B. tabaci} after substitution of glutamine with lysine, histidine with glutamine, and leucine with methionine at amino acid residues at 124, 149 and 174, respectively [96]. Further, Pan et al., while examining the key amino acid residues responsible for differential transmission of squash leaf curl China virus (SLCCNV), found that a threonine to serine substitution at residue 147 altered the SLCCNV transmission by \textit{B. tabaci Asia 1} and \textit{B. tabaci Asia II} [95]. An alignment of the amino acid sequences of the capsid protein...
of B. tabaci MED transmissible and non-transmissible begomoviruses did not reveal any consistent patterns in this study. However, at amino acid residue location 85, all B. tabaci MED non-transmissible viruses except for tomato yellow leaf curl China virus (TYLCCV) had valine, whereas B. tabaci MED-transmissible viruses had isoleucine (Figure 6). Whether this change is just a random occurrence or if it plays any role in determining the interactions between begomoviruses and B. tabaci MED is not clear and warrants further investigation. These results suggest that the inability of B. tabaci MED to transmit the two NW bipartite viruses could possibly be due to random mutations within the capsid protein or other vector determinants.

5. Conclusions

The current study showed that the circulative movement of OW (TYLCV) and NW (CuLCrV and SiGMV) begomoviruses within B. tabaci MED differed in their ability to cross the midgut barrier. During circulative movement within B. tabaci, begomoviruses interact with many vector receptors, immune proteins, and/or symbionts’ proteins located at MG, hemolymph, and PSG [48,98–100]. For instance, two B. tabaci MEAM1 midgut proteins, viz., BtCUBN and amnionless (BtAMN), form a BtCubam receptor complex that binds to the capsid protein of TYLCV, resulting in clathrin-mediated endocytosis of TYLCV into whitefly midgut cells [101]. In the present study, both B. tabaci MED and B. tabaci MEAM1 efficiently transmitted TYLCV, but only B. tabaci MEAM1 was able to transmit CuLCrV or SiGMV. Therefore, it is very likely that factors required for efficient circulation of TYLCV within whiteflies are present in both B. tabaci MED and B. tabaci MEAM1. However, factors required for efficient circulation of CuLCrV or SiGMV were present only in B. tabaci MEAM1. Thus, it is possible that different begomoviruses interact with different whitefly transmission determinants and these factors could differ between cryptic species. Currently, these whitefly factors remain incompletely understood and warrant further investigation. Moreover, at this time it is not clear whether the longer association between NW begomoviruses with B. tabaci MEAM1 (30–40 years) in comparison with B. tabaci MED (~15 years) provided an opportunity for the NW begomoviruses and B. tabaci MEAM1 to coevolve favoring transmission, or whether these differential interactions are a result of innate transmission determinants. From a limited number of studies conducted, it seems that B. tabaci MED readily transmits OW begomoviruses, whereas B. tabaci MEAM1 seems to be an effective vector of both OW and NW begomoviruses. The establishment of B. tabaci MED in the United States can have severe impacts on OW begomovirus epidemics, such as TYLCV in tomato production. However, similar trends may not be observed for NW begomoviruses, such as CuLCrV and SiGMV, with the caveat being that this study was conducted only with these two NW begomoviruses and under controlled conditions.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/v14051104/s1. Table S1: Validation of insects and plant samples surface sterilization; Table S2: Infection or non-infection of cotton with TYLCV, CuLCrV, or SiGMV; Table S3: Primary and secondary endosymbionts within B. tabaci MEAM1 and B. tabaci MED populations; Figure S1: TYLCV, CuLCrV, or SiGMV accumulation in tomato, squash, or prickly sida.

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