INFECTIOUS CLONES DEVELOPMENT AND TRANSMISSION BIOLOGY OF MAIZE CHLOROTIC MOTTLE VIRUS

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Abstract

Maize chlorotic mottle virus (MCMV) has become one of the most widespread corn viruses in the Hawaiian Islands. In the US Mainland, MCMV has been reported to be transmitted by chrysomelid beetles. However, none of these beetle species are established in Hawaii where the corn thrips, *Frankliniella williamsi* has been identified to be the main vector. In this research, I showed that thrips transmitted the virus with no evidence for latent periods. Both larvae and adults transmitted MCMV for up to 6 days after acquisition, with decreasing rates of transmission as time progressed. There was also no evidence that adult thrips that acquired the virus as larvae were competent vectors. Real-time RT-PCR assays showed that viral load was depleted from the vector's body after thrips had access to healthy plant tissue. Depletion of viral load was also observed when thrips matured from larvae to adults. Thrips were able to transmit MCMV after acquisition and inoculation access periods of 3 hours. In addition, I used an artificial feeding assay to feed thrips on purified MCMV or MCMV genomic RNA. Based on results achieved, I postulated that MCMV employs a capsid strategy for vector transmission. To further study the molecular determinants in MCMV transmission by vectors, I developed infectious clones of MCMV using a long RT-PCR assay. Taken altogether this research suggests that corn thrips transmit MCMV in a semi-persistent manner and that MCMV employs a capsid strategy for vector transmission.

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Chapter 1. Introduction

Plant virus diseases often have tremendous negative impacts on the economy and subsistence of agricultural commodities. The most epidemic plant virus diseases attain high infection rates and spread rapidly when transmitted by arthropod vectors, particularly those belonging to three major orders: Hemiptera, Thysanoptera, and Coleoptera (Nault 1997). The order Hemiptera contains the most important lineages of virus vectors including the aphids, the whiteflies, the leafhoppers, and the planthoppers.

Seed corn production has become Hawaii's most valuable agricultural commodity, worth $247.2 million in 2011 (Hawaii Seed Crops, NASS). The re-appearance of *Maize chlorotic mottle virus* (MCMV) (*Tombusviridae*: *Machlomovirus*) has become a major threat for Hawaii's seed corn industry. Corn plants infected with MCMV show symptoms of chlorotic mottling on leaves and stunted growth. When MCMV-infected corn plants are co-infected with either *Maize dwarf mosaic virus* (MDMV), *Sugarcane mosaic virus* (SCMV), or *Wheat streak mosaic virus* (WSMV), a synergistic effect between multiple viral infections results in a severe disease called Corn Lethal Necrosis (CLN), which has been reported to reduce crop yield by up to 90-100% (Goldberg and Brakke 1987, Niblett and Claflin 1978, Uyemoto et al. 1980). The potential yield losses of such an outbreak will be devastating to the corn seed industry and to Hawaii's agricultural economy.

On the US mainland, MCMV is vectored by Chrysomelid beetles (Coleoptera) (Nault et al. 1978), none of which currently occur in the Hawaiian Islands. The only known vector of MCMV in Hawaii is the corn thrips *Frankliniella williamsi* (Thysanoptera), a phylogenetically unrelated species (Jiang et al. 1992). In general, plant viruses belonging to the same genus are transmitted by specific vector taxa (Nault 1997, Ng and Falk 2006). For example, the plant virus

genus *Potyvirus* (family *Potyviridae*) is comprised of at least 118 species, all of which are transmitted by aphids (Raccah et al. 2001, Ng and Falk 2006). The same holds true for the 14 virus species comprising the genus *Tospovirus* (*Bunyaviridae*), all of which are transmitted by thrips (Whitfield et al. 2005). In most cases, plant viruses that are transmitted by leaf-feeding beetles are not transmissible by other arthropod vectors (Gergerich 2001). These trends of association are commonly observed across several plant virus genera and are likely the result of a process of co-evolution (Nault 1997). Therefore, the transmission of MCMV by both corn thrips and Chrysomelid beetles is significant since the virus is vectored by phylogenetically unrelated species, with a distinct developmental biology, ecology and feeding strategies.

The relationship of plant viruses and their insect vectors is complex, and governed by several determinants: the time insects require to acquire the virus from the infected plants (acquisition), the time insects require to inoculate the virus to healthy hosts (inoculation), the time insects retains the virus after acquisition (persistence), the time lapse between acquisition and inoculation (latent period), and the retention of infectivity through insect development (transtadial passage) (Ng and Falk 2006). These parameters have permitted the characterization of virus transmission by arthropod vectors into 4 broad categories: non-persistent, semi-persistent, circulative, and propagative (Hogenhout et al. 2008, Ng and Falk 2006).

In addition, several studies have revealed that vector-virus specificity is regulated at the molecular and cellular level (Gergerich 2001, Hogenhout et al. 2008, Ng and Falk 2006). For several aphid-transmitted plant viruses, transmission is determined by successful binding of the viral particles to insect vector's receptors. Virus particles transmitted non- and semi-persistently can bind to the stylets either directly (capsid strategy) or indirectly through viral proteins, which have been broadly called helper factors (helper factor strategy), which bridge the virus to the

insect's receptors (Ng and Falk 2006). Because the mode of transmission has important epidemiological implications, determining these factors for MCMV transmission may lead to better pest management practices.

The goal of my research is to gain understanding of the way MCMV and the corn thrips interact for transmission. Specifically, I have worked toward 3 objectives, each of which have been developed in distinct chapters: (1) determine the mode of transmission of MCMV by the corn thrips (2) define whether MCMV is transmitted using a helper factor or capsid strategy, (3) develop infectious clones that can be used to further examine the molecular determinants for the virus-vector interaction.

Chapter 2. Transmission mode of MCMV by the corn thrips

2.1 Introduction

Maize chlorotic mottle virus (MCMV) (*Tombusviridae*: *Machlomovirus*) is a plant virus with icosahedral virions of 30 nm in diameter containing a single-stranded positive-sense RNA of approximately 4.4 kb (Nutter et al. 1989, Lommel et al. 1991, Stenger and French 2008). MCMV does not infect dicots but has a wide monocot host range with at least 19 grass species including cultivated ones, such as barley (*Hordeum vulgare* L.), proso millet (*Panicum miliaceum* L.), foxtail millet (*Setaria italica* L.), and wheat (*Triticum aestivum* L.) (Bockelman et al. 1982). However, to date MCMV causes the most significant economic yield losses to corn (*Zea mays* L.).

MCMV was first described in Peru (Castillo and Hebert 1974) and its current distribution spans several countries in North and South America, including: Argentina (Teyssandier et al. 1983), Brazil, Mexico (Gordon et al. 1984), and USA (Niblett and Claflin 1978, Uyemoto et al. 1980, Jiang et al. 1992). Recently, the virus has also been recorded in some Asian countries, such as China (Xie et al. 2011) and Thailand (Scheets 2008). In the US, MCMV is present in Kansas (Niblett and Claflin 1978), Nebraska (Doupnik 1979), Texas (Nyvall 1999), and Hawaii (Jiang et al. 1992). Since MCMV is transmitted at low rates (0.008-0.04%) through the corn seeds (Jensen et al. 1991), it has been suggested the trades of MCMV-infected seeds has contributed to the wide distribution of this virus (Jensen et al. 1991, Scheets 2008).

Corn plants infected by MCMV show symptoms of chlorotic mottling on leaves, and stunting in growth. It has been reported that MCMV infection can reduce crop yield by 10-15% (Castillo and Hebert 1974, Loayza 1976, Nault et al. 1981). When MCMV co-infects corn plants with other corn viruses from the family *Potyviridae*, such as *Maize dwarf mosaic virus* (MDMV)

(genus: *Potyvirus*), *Sugar cane mosaic virus* (SCMV) (genus: *Potyvirus*), or *Wheat streak mosaic virus* (WSMV) (genus: *Tritimovirus*), their synergistic effect causes a more severe disease called Corn Lethal Necrosis (CLN) (Uyemoto et al. 1980, Goldberg and Brakke 1987). Symptoms of CLN include leaf necrosis, severe plant stunting, and premature death. CLN-infected plants have average MCMV concentrations 3-11 times higher than singularly infected MCMV plants (Goldberg and Brakke 1987; Scheets 1998). It has been reported that CLN disease can reduce crop yield by up to 90% (Niblett and Claflin 1978; Uyemoto et al. 1980).

Nault and colleagues (1978) showed that six species of Chrysomelid beetles are vectors of MCMV. These include: the western corn rootworm *Diabrotica virgifera virgifera* LeConte, the northern corn rootworm *D. barberi* Smith & Lawrence, the southern corn rootworm *D. undecimpunctata howardi* Barber, the corn flea beetle *Chaetocnema pulicaria* Melsheimer, the flea beetle *Systena frontalis* (Fabricius), and the cereal leaf beetle *Oulema melanopus* (L.). Eight other potential vector species screened by Nault and colleagues (1978), including the Japanese beetle *Popillia japonica* Newman, the black cutworm *Agrotis ipsilon* (Hufnagel), the bird cherry-oat aphid *Rhopalosiphum padi* L., the green peach aphid *Myzus persicae* (Sulzer), the greenbug *Schizaphis graminum* (Rodani), the whitefly *Trialeurodes vaporariorum* Westwood, the corn planthopper *Peregrinus maidis* (Ashmead) and the blackfaced leafhopper *Graminella nigrifrons* (Forbes), failed to transmit the virus. The beetle species *D. viridula* (Fabricius) and *D. decempunctata* (Latreille) were also identified as vectors of MCMV in Peru (Castillo 1983).

Transmission of MCMV by chrysomelid beetles resembles that of many other plant viruses transmitted by beetles (Gergerich 2001). For instance, Nault et al., (1978) found that MCMV was transmitted immediately after acquisition and persisted for up to six days in the western corn rootworm and in the cereal leaf beetle. In addition, there was no evidence of transstadial passage

since larvae of the cereal leaf beetle lost the ability to transmit the virus after adult eclosion. Larvae of the cereal leaf beetle were more efficient vectors of MCMV than their adult counterparts. Jensen (1985) performed additional transmission experiments by using three corn rootworm species: the southern, the northern, and the western corn rootworm. The author did not find evidence of a latent period nor of transovarial passage. Furthermore, he found that MCMV had to be acquired through insect feeding, and that surface contamination alone was not enough to support transmission. Larvae were shown to effectively transmit MCMV, which was recovered in some trace amounts, in the insect's gut and hemolymph (Jensen 1985).

Because none of the chrysomelid species vectors of MCMV occurred across the Hawaiian Islands (Nishida 2002), Jiang and colleagues (1992) conducted an additional study aimed at identifying potential arthropod vectors of MCMV in cornfields cultivated in the island of Kauai (Hawaii, USA). Of six arthropod species tested: *P. maidis*, *Sardia pluto* (Kirkaldy), the southern garden leafhopper *Empoasca solana* DeLong, the Chinese rose beetle *Adoretus sinicus* Burmeister, mites of the genus *Tetranychus* spp. and the corn thrips *Frankliniella williamsi* Hood, the authors found that the sole species capable of transmitting MCMV was the corn thrips, *F. williamsi* (Jiang et al. 1992).

To date, it is not known through which mode the corn thrips may transmit MCMV. Currently, thrips are known to transmit plant viruses belonging to different genera: *Tospovirus, Ilarvirus*, *Carmovirus*, and *Sobemovirus* (Jones 2005, Whitfield et al. 2005). These viruses can be transmitted by thrips in different ways. For instance, *Tomato spotted wilt virus* (TSWV) (*Bunyaviridae*: *Tospovirus*) is transmitted by the western flower thrips, *Frankliniella occidentalis* (Pergande) and other thrips species in a propagative-persistent manner (Wijkamp et al. 1996, Whitfield et al. 2005). TSWV is generally acquired by the first instar larvae and is

transmitted after adult eclosion (Wijkamp and Peters 1993, Ullman et al. 1992). The replication of the virus within the thrips body ensures the persistence of transmission over long periods of time (Whitfield et al. 2005). Other viruses, like *Tobacco streak virus* (TSV) (*Bromoviridae: Ilarvirus*), *Pelargonium flower break virus* (PFBV) (*Tombusviridae: Carmovirus*), or *Sowbane mosaic virus* (SoMV) (*Sobemovirus*) are transmitted by the thrips as a sort of "mechanical accident" (Krczal et al. 1995, Hardy and Teakle 1992). All of these viruses seem to be pollen-borne (Jones 2005). For PFBV and SoMV, it has been shown that thrips may transport virus-infected pollen from infected plants to healthy ones and facilitate inoculation through feeding wounds. Thrips may also facilitate the transmission of virus by introducing through feeding the infected pollen that cover the leaf surface, as demonstrated for TSV (Reddy et al. 2002, Jones 2005). None of these viruses seem to directly interact with the thrips vectors.

In this work I examined the mode of MCMV transmission by the corn thrips. I tested the competence of virus transmission by larvae and adults, the persistence of transmission, and the possible retention through thrips development. I adopted a real-time reverse transcription polymerase chain reaction (RT-PCR) assay to quantify the change in viral load in thrips over time and throughout their development. Finally, I tested the variation of MCMV acquisition and inoculation efficiency over time.

2.2 Materials & Methods

Thrips rearing and production of corn seedlings. A colony of corn thrips was established in September 2009 from adults collected from corn ears harvested in a sweet corn field (cv. Golden Cross Bantam (Wetsel Inc., Harrisonburg, VA) on the eastern side of Oahu (Hawaii, USA).

Thrips were transferred to potted corn seedlings cv. Golden Cross Bantam inside cylindrical mesh ventilated PVC cages (1016 mm tall, 120 mm in diameter) in a greenhouse with a

photoperiod of approximately 12 h and a temperature range of 27 ± 5°C. To produce coetaneous insects, about 40-50 adults were transferred to caged three-week-old corn seedlings and were allowed to lay eggs for one week. Adults were thereafter removed from cages and the eggs laid were allowed to develop into adults, which appeared in approximately three to four weeks. Newly emerged adults were periodically used for colony maintenance whereas other adults or larvae were used for the transmission assays as described hereafter. Leaf samples from the corn seedlings used for thrips rearing were periodically tested by double antibody sandwich enzyme-linked immunosorbant assay (DAS-ELISA) for MCMV to ensure the insect colony was not infected.

Corn seedlings of cv. Golden Cross Bantam used to rear thrips and for the transmission assays were sown in a soil mix made up of 3 parts of Sun Grow Sunshine® Mix #4 (Sun Gro Horticulture, Vancouver, Canada), 1 part of Perlite (Supreme Perlite Company, Portland, OR), and 2 parts of Vermiculite (Therm-O-Rock West Inc., Chandler, AZ) inside 127 mm diameter plastic pots. Plants were fertilized bimonthly with 36-6-6 Miracle-Gro® Lawn Food (The Scotts Company, Marysville, OH). Plants were maintained inside insect-proof cages in a greenhouse with a photoperiod of approximately 12 h and a temperature range of 27 ± 5 °C. They were kept in a separated section of the greenhouse to avoid uncontrolled thrips infestation or MCMV contamination.

MCMV characterization and maintenance. Leaves from corn plants showing typical MCMV mottling symptoms were collected from a corn field at a Pioneer Hi-Bred station at Kekaha (Kauai, Hawaii, USA) in December of 2009. MCMV was transferred to corn seedlings by mechanical inoculation. The virus was characterized by ELISA assays on both the original field infected leaves as well as on the mechanically inoculated seedlings. The plant tissue was

screened by ELISA against 6 different corn viruses: *Maize mosaic virus* (MMV) (*Rhabdoviridae*: *Nucleorhabdovirus*), MCMV, WSMV, MDMV, SCMV as well as with antibodies that react against the entire Potyvirus genus (AGDIA, Elkhart, IN).

In addition, I sequenced our laboratory isolate of MCMV (MCMV-HI) (4.4 kb) using an approach similar to that described by Stenger and French (2008). Genomic sequences obtained possessed a very high nucleotide similarity, over 97%, to the Nebraska and Kansas strains (results reported in chapter 4). MCMV was transferred monthly to new corn seedlings via mechanical inoculation. Plants were maintained inside insect-proof cages in a greenhouse with a photoperiod of approximately 12 h and a temperature range of 27 ± 5°C.

MCMV transmission to leaf disks and to corn seedlings. In preliminary experiments, I compared the transmission of MCMV to corn seedlings or to leaf disks. Healthy adults, starved for two hours, were transferred into Tashiro cages (Tashiro, 1967) containing a portion of excised MCMV-infected corn leaf. After an acquisition access period (AAP) of 48 h, 20 thrips were individually confined to one-week-old corn seedlings inside mesh-ventilated cylindrical, PVC tubes (30 cm tall, 7.5 cm diameter). After an inoculation access period (IAP) of 48 h, the corn seedlings were sprayed with Imidacloprid insecticide (Bayer Advanced, Research Triangle Park, NC) at a concentration of 0.012% and were transferred to a greenhouse for symptom development. Alternatively**,** MCMV transmission was tested using a modified leaf disk assay (Wijkamp and Peters 1993). Leaf disk chambers were created from 15-ml Falcon tubes by transversely cutting tubes 15 mm below the lid. Leaf disks, 14 mm in diameter, were excised from healthy corn leaf midribs. One milliliter of melted 1% agar solution was poured into the cap and a leaf disk was placed on the surface before the agar solidified. After an AAP of 48 h, 20 thrips were individually confined to leaf disk chambers. Chambers were sealed with stretched

Parafilm® and pierced on the top with a pin to allow ventilation. At the end of an IAP of 48 h, thrips were removed from the chambers and transferred into 1.5 ml tubes containing 70% ethanol. The leaf disks were incubated in their chambers at 25 ± 2°C for another four days to allow the virus to replicate. Leaf disks were thereafter removed from the chamber, cleaned from the agar by washing under tap water, and processed for ELISA to test for the presence of MCMV as described below. Five uninfected thrips collected from rearing colonies were used as controls. Transmission to either leaf disks or seedlings were replicated 3 times. For statistical analysis, the proportion of MCMV-infected leaf disks or seedlings were transformed by arcsine root square and compared with a *t*-test using the statistical software SigmaStat Version 3.5 (San Jose, CA).

Persistence. Persistence experiments used the previously described leaf disk assay with some modification. Healthy adults, starved for two hours, were transferred to Tashiro cages containing excised MCMV-infected corn leaves. After an AAP of 48 h, thrips were starved for two hours and individually transferred to healthy corn leaf disks for an IAP of 48 h. Each thrips was thereafter serially transferred every 48 h to new leaf disks. Experiments lasted for the entire lifespan of the thrips. Leaf disks were handled as previously described. DAS-ELISA tests were performed on the leaf disks to test for the presence of MCMV. Persistence experiments were replicated twice.

Virus retention through thrips development. To examine the retention of MCMV through thrips development, second instar larvae were collected from rearing cages and were starved for two hours before being transferred to Tashiro cages, where they were allowed to feed on excised MCMV-infected corn leaves for an AAP of 48 h or until reaching their pupal stage. Pupae were then individually transferred to healthy corn leaf disks for a period of 48 h, thrips were serially transferred for 2-3 passages to healthy leaf disks with an IAP of 48 h each. DAS-ELISA tests

were performed on the leaf disks to test for the presence of MCMV. Real-time RT-PCR described below was used to quantify viral load in thrips.

Transmission efficiency. Adults starved for two hours were transferred to Tashiro cages containing excised MCMV-infected corn leaves. Five batches of 20 thrips were allowed to feed for AAPs of 3, 6, 12, 24 or 48 h. Insects were collected from the cages at the end of each assigned acquisition period, starved for another two hours, then individually transferred to healthy corn leaf disks for an IAP of 48 h. At the end of the IAP, thrips were removed and transferred into 1.5 ml tubes containing 70% ethanol, whereas the leaf disks were handled as previously described to allow for virus replication. A similar procedure was used for another series of transmission experiments where batches of 20 thrips were allowed to acquire MCMV by feeding on infected leaves for AAP of 48 h followed by IAPs of 3, 6, 12, 24 or 48 h. Additional tests were conducted to examine the possibility that thrips may transmit MCMV similarly of aphid-transmitted non-persistent viruses (Ng and Falk 2006). For these assays, two-hour starved adult thrips were allowed to feed on MCMV-infected corn leaves for an AAP of 10 min, then batches of 3 insects were transferred to healthy corn leaf disks for an IAP of 1 h. These experiments were replicated 3 times. DAS-ELISA tests were performed on the leaf disks to detect for the presence of MCMV. Transmission rates were plotted against time and were fitted to linear regressions approximated using least squares approach of the statistical software SigmaStat Version 3.5 (San Jose, CA).

ELISA. Leaf disks used in transmission assays were tested for MCMV by DAS-ELISA. Leaf extracts were prepared by grinding each leaf disk with pestles in a 1.5 ml tube containing 300 µl of extraction buffer (10.3 mM Na_2SO_3, 2% Polyvinylpyrrolidone, 3.1 mM NaN_3, 0.2% powdered chicken egg albumin, and 2% Tween 20, pH 7.4). ELISA was performed according to

manufacturer's instructions (AGDIA, Elkhart, IN, USA). After color development, a reading of absorbance values was taken at A_{405nm} using a microplate reader (Model 680, Bio-Rad, Hercules, CA). Positive readings were determined as values greater than twice the average reading of the negative controls. MCMV-infected and healthy corn leaf samples were used as positive and negative controls, respectively. ELISA tests were used to confirm for the presence of MCMV in mechanically inoculated corn tissues used for the transmission tests.

RNA Extraction and Reverse Transcription. RNA was isolated from leaf disks excised from infected or from healthy seedlings as well as from thrips produced for the persistence and the retention through thrips development experiments using an RNeasy Mini Kit (Qiagen, Valencia, CA). Leaf disks or groups of three thrips were ground in a 1.5 ml tube containing 350 µl buffer RLT. Samples were vortexed for 5 minutes and then centrifuged in QIAshredder columns (Qiagen, Valencia, CA) for 2 minutes, high speed. RNA extraction was performed according to manufacturer's instructions (Qiagen, Valencia, CA). Samples were then reverse transcribed using a QuantiTect Reverse Transcription Kit (Qiagen, Valencia, CA).

Real-Time RT-PCR. I adopted a real-time RT-PCR assay to quantify MCMV in thrips bodies using a similar procedure adopted by Rotenberg et al. (2009) to quantify TSWV within the western flower thrips. A relative quantification was done by targeting the MCMV open reading frame encoding for the coat protein, and as a reference gene, the thrips elongation factor-1 alpha (EF-1α). The universal EF-1α gene primers Manto (5′-GGAACBTCWCAGGCTGAYTGTGC-3′) and Phasma (5′-GGCGCRAADGTNACNACCATDCCRGG-3′) described by Djernaes & Damgaard (2006) were used to obtain a 532 bp from the EF-1α gene of the corn thrips. The amplicon obtained was sequenced from the 5′ and 3′ ends and used with the software Primer3 (v. 0.4.0) to design a primer pair specific to the corn thrips EF-1α gene, EF_fwf (5′-

TCTCCAAGAACGGACAGACC-3′) and EF_Fwr (5′-GATCAGCTGCTTCACACCAA-3′). Nucleotide sequences generated from MCMV-HI were used to design the primer pair MCMV_CPf (5′-TTAACTCTGTGCGCATCACC -3′) and MCMV_CPr (5′-ATCCGCTAGTGGTGTCTGCT -3′). The specificity of the primer pairs were tested in PCR assays and were confirmed by sequencing the amplicons obtained as well as in melting curve analysis.

Real-time RT-PCR reactions were prepared with a master mix containing 10 µl of SYBR® Green (Qiagen, Valencia, CA), 1 µl of 10 µM of each primer, and 2.5 µl of cDNA in a final volume of 20 µl. Samples were amplified in duplicates using a Rotor-Gene™ 6000 Thermal Cycler (Corbett Research, San Francisco, CA). The relative amount of MCMV cDNA was normalized to the amount of EF-1α cDNA using the equation described by Pfaffl (2001): $E^{Ct\,(EF\text{-}1\alpha)} / E^{Ct\,(MCMV)}$; where E = PCR efficiency of a given primer pair, and Ct = cycle threshold, which was automatically calculated by the PCR machine's software (Rotor-Gene 6000 Series Software Version 1.7, Corbett Research, San Francisco, CA). Efficiency values were calculated by using the formula: $E = 10^{(-1/slope)}$. The slopes of the curves were determined empirically by four-fold serial dilutions. Since E = 2.00 was achieved for both MCMV and EF-1α primer pairs, I adopted a simplified equation: $2^{\,Ct\,(EF\text{-}1\alpha - MCMV)}$ (Pfaffl 2001) to calculate the relative amount of MCMV in each sample. To analyse data statistically, the relative concentrations of MCMV within thrips were replicated 5 times, each consisting of 3-pooled thrips. Data were examined using a Kruskal-Wallis test. Pairwise comparisons were tested for differences adopting a Mann-Whitney U test. Both statistical tests were performed with the software SigmaStat Version 3.5 (San Jose, CA).

2.3 Results

Transmission of MCMV to seedlings and leaf disks. No statistical differences were observed in the rates of MCMV transmission by individual thrips using corn seedlings or leaf disks, which averaged at 0.37 ± 0.08 (SE) and at 0.39 ± 0.08 (SE), respectively ($t = 0.17$; df = 4; $P = 0.87$). Therefore, leaf disks were used as a routine technique for the subsequent transmission assays.

Persistence. Adults were allowed to feed for 48 h on MCMV-infected leaves followed by serial passages to healthy leaf disks on which individual thrips fed for 48 h IAPs. Out of 52 thrips tested in 2 replicates, 22 (42%) were able to transmit MCMV (Fig. 1A). When virus transmission was examined over time, I observed a decrease in the rate of transmission (Fig. 2). The virus was not transmitted by thrips beyond 6 days post-acquisition (Fig. 1A). Of the 22 transmitters, 21 (95%) inoculated MCMV to only 1 leaf disk, and 16 out of 22 (73%) thrips transmitted MCMV to the first leaf disk used in the serial passages.

Real time RT-PCR showed a decrease in the average concentration of MCMV in the thrips (Fig. 3A). Significant differences were observed when the Kruskal-Wallis test was applied ($H = 16.45$; df = 4; $P = 0.002$). The titer of MCMV relative to the thrips gene EF-1α peaked at the end of the 2 days AAP (reported in Figure 3A as days 0) at averages of 74.3 to only 1.3 after thrips had access to healthy leaves for an additional 2 days ($P = 0.016$; Mann-Whitney test) thus evidencing depletion of the virus from the thrips.

Virus retention through thrips development. Out of 54 second instar larvae tested in 2 replicates, 24 (44%) were able to transmit MCMV (Fig. 1B). However, the pupae and the emerging adults that developed from those larvae were no longer able to transmit the virus (Fig. 1B). Similarly, when 41 larvae, tested in 2 replicates, were allowed to complete their development on MCMV-infected leaves, the pupae and adults that were subsequently transferred

to the leaf disks were unable to transmit the virus (Fig. 1C). Real time RT-PCR showed a decrease in the average concentration of MCMV in the thrips (Fig. 3B). Significant differences were detected when the Kruskal-Wallis test was applied ($H = 12.95$; df = 4; $P = 0.012$). The relative titer of MCMV in the larvae was 10-30 times larger than what I observed into the pupae or emerging adults ($P < 0.05$; Mann-Whitney test), indicating a rapid depletion of the virus during the thrips development. As shown by contrasting Fig. 3A and 3B, viral load into the adults was 6 times larger than what observed into the larvae ($P < 0.05$; Mann-Whitney test).

Transmission efficiency. None of the 60 adult thrips, which were tested for transmission in batches of 3, were able to transmit MCMV by feeding on virus-infected corn leaves for an AAP of 10 min followed by feeding on healthy corn leaf disks for an IAP of 1 h, suggesting MCMV is not vectored with a non-persistent transmission mode. However, individual adult thrips were able to transmit MCMV after AAP and IAP of 3 h (Fig. 4). In addition, the transmission efficiency increased when thrips had longer AAPs and IAPs following linear regressions over time (Fig. 4).

2.4 Discussion

To my understanding this is the first study reporting experimental evidence on the mode of MCMV transmission by the corn thrips. For most of our transmission assays I elected to condition insects to a fasting period since I noticed thrips spent a lot of time walking into the chambers rather than attempt feeding on the leaf disks. I also elected to use IAPs of 2 days since this was shown in our preliminary transmission assays to support high rates of MCMV transmission. Furthermore, transmission and inoculation access periods ranging from 1 to 4 days have been previously adopted to examine the transmission of MCMV by the chrysomelid beetles (Nault et al. 1978, Jensen 1985). In addition, I tested MCMV transmission under acquisition and

inoculation times required by aphid-transmitted non-persistent viruses, however I failed to obtain evidence of virus transmission under these experimental conditions.

Tomato spotted wilt virus, which propagates within the thrips vectors, is generally acquired by the larvae and then is persistently transmitted after adult eclosion (Wijkamp and Peters 1993, Ullman et al. 1992). The pattern of TSWV transmission contrasts with the pattern of transmission observed with MCMV. In fact, the corn thrips larvae lose the virus soon after feeding on healthy plant tissues. Even when the second instar larvae are allowed to complete their development on MCMV-infected leaves, neither the pupae nor the emerging adults are competent in transmitting the virus. These results are important under an epidemiological point of view as they suggest that MCMV likely spread through the vector activity of adult thrips, which are responsible for both phases of virus acquisition and inoculation. Since larvae have a limited dispersal activity, they likely have a limited role in vectoring MCMV even when they develop from virus-infected plants.

The pattern of MCMV transmission by the corn thrips closely resembles those for the same virus by Chrysomelid beetles (Nault et al. 1978, Jensen 1985). Similar to the western corn rootworm, thrips transmit MCMV soon after acquisition and retain the virus for only a few days with no evidence for a latent period. As in the case of the chrysomelid beetle vectors of MCMV, the corn thrips is able to efficiently transmit the virus as larvae and adults with similar transmission efficiency (Gergerich 2001, Nault et al. 1978). The short retention times, and the lack of virus retention through the thrips development, are also the characteristics of MCMV transmission by the corn thrips and the Chrysomelid beetles.

Quantification of MCMV by real time-RT PCR showed a depletion of the viral load after thrips were transferred to feed on the healthy corn tissues. Similarly, thrips lost the entire viral

load through their development from larvae to adults. Although both larvae and adults were competent in vectoring MCMV, the viral load in adults was determined to be at least 6 times greater than what observed in larvae. Altogether, the data obtained by real-time RT-PCR is consistent with the results from transmission assays. Collectively, these results suggest that MCMV is transmitted semi-persistently by the corn thrips. Semi-persistent viruses generally persist in their vectors for a few days, they are not retained after the moulting, and have no detectable latent period (time between acquisition and inoculation) in their vectors (Ng and Falk 2006). To my knowledge, this is the first work reporting evidence of a plant virus transmitted semi-persistently by thrips.

To date is unclear why the MCMV is not retained through the thrips development; both thrips moulting and time passing can contribute to the observed depletion of virus into the insect. Future additional assays that consider shorter feeding time may shed lights to the reasons underling the lack or virus retention.

Because field sampling has revealed the occurrence of different thrips species in cornfields in Hawaii (unpublished), the question is raised whether other thrips species may be competent MCMV vectors. From an epidemiological perspective it would be interesting to understand whether thrips may be implicated in the epidemiology of MCMV in corn growing areas other than Hawaii. For instance, in Nebraska and Kansas there have been indication that MCMV spreads very rapidly during the maize-growing season, suggesting that beetles may not the major source of virus spread (Scheets 2008). Although MCMV tends to reappear in previously infected fields, the virus has not been demonstrated to be soil, water or fungus-borne. Recently, outbreaks of MCMV has been reported in corn-growing regions of Yunnan - China (Xie et al. 2011). However, to my understanding it is not known what vector species may be implicated.

In summary, my research provides evidence that MCMV is transmitted by corn thrips semi-persistently. This appears to be a unique feature among thrips-vectored plant viruses.

Chapter 3. MCMV uses a capsid strategy for transmission by the thrips vector

3.1 Introduction

I have previously shown that the pattern of *Maize chlorotic mottle virus* (MCMV) (*Tombusviridae*: *Machlomovirus*) transmission by the corn thrips *Frankliniella williamsi* (Thysanoptera) closely resembles that of the same virus by chrysomelid beetles (Coleoptera) (Nault et al. 1978, Jensen 1985). Similar to the western corn rootworm*, Diabrotica virgifera virgifera* LeConte, thrips transmit MCMV soon after acquisition and retain the virus for only a few days with no evidence for a latent period. As in the case of the chrysomelid beetle vectors of MCMV, the corn thrips is able to efficiently transmit the virus as larvae and adults with similar transmission efficiency (Cabanas et al. 2013, Gergerich 2001, Nault et al. 1978). The short retention times, and the lack of virus retention through the thrips development, are also characteristic of MCMV transmission by the corn thrips and the chrysomelid beetles. In summary, my previous research provides evidence that MCMV is transmitted by corn thrips semi-persistently.

In general, hemipteran vectors retain non- and semi-persistent viruses in the mouthpart or foreguts (Chen et al. 2011, Ng and Falk 2006, Uzest et al. 2011). Virus-encoded determinants mediate virus binding by specifically interacting with the vector receptors located on the insect's stylet or foreguts. Binding is mediated either directly by the virus coat protein (capsid strategy) or indirectly though virus-derived non-structural proteins (helper factor strategy) (Ng and Falk 2006). The helper factor strategy is used by aphid-transmitted Potyviruses (Potyviridae) and Caulimoviruses (Caulimoviridae). Alternatively, the capsid protein of a virus particle can interact

directly with the insect receptor, as in the case of the aphid-transmitted *Cucumber mosaic virus* (CMV) (Cucumoviridae). For instance, in early heteroencapsidation experiments, when coat proteins of an aphid transmissible isolate of CMV was mixed with RNA from a CMV isolate that was poorly transmitted by aphids, aphid transmission of the assembled virions was restored (Gera et al. 1979, Ng and Falk 2006). Using infectious clones and side-directed mutagenesis assays, more recent studies identified the residues in the coat protein that affected the transmission of CMV by the aphid species: *Aphis gossypii* and *Myzus persicae* (Perry et al. 1994, Ng et al. 2005, Ng and Falk 2006). Changes in the amino acid sequence of CMV has 2 possible effects: (1) alteration of the physical structure of the coat protein that results in an increased instability of the virions and a consequent reduction in transmission rates; and (2) changes in the transmission followed modifications in the residues in the coat protein that directly bind to putative receptors in the vector stylets (Ng et al 2005, Ng and Falk 2006).

In this chapter, I examined whether MCMV is transmitted using a helper factor or capsid strategy. To achieve this goal, I purified MCMV and developed an artificial feeding assay to allow corn thrips to feed on purified MCMV virions or infectious MCMV genomic RNA. After thrips were allowed to feed on the virus preparations, they were confined to healthy leaf seedlings to test the transmission.

3.2 Materials & Methods

Thrips rearing and production of corn seedlings. A colony of corn thrips was established in September 2009 from adults collected from corn ears harvested in a sweet corn field (cv. Golden Cross Bantam (Wetsel Inc., Harrisonburg, VA) on the eastern side of Oahu (Hawaii, USA).

Thrips were transferred to potted corn seedlings cv. Golden Cross Bantam inside cylindrical mesh ventilated PVC cages (1016 mm tall, 120 mm in diameter) in a greenhouse with a

photoperiod of approximately 12 h and a temperature range of 27 ± 5°C. To produce coetaneous insects, about 40-50 adults were transferred to caged three-week-old corn seedlings and were allowed to lay eggs for one week. Adults were thereafter removed from cages and the eggs laid were allowed to develop into adults, which appeared in approximately three to four weeks. Newly emerged adults were periodically used for colony maintenance whereas other adults were used for the transmission assays as described hereafter. Leaf samples from the corn seedlings used for thrips rearing were periodically tested by double antibody sandwich enzyme-linked immunosorbant assay (DAS-ELISA) for MCMV to ensure the insect colony was not infected.

Corn seedlings of cv. Golden Cross Bantam used to rear thrips and for the transmission assays were sown in a soil mix made up of 3 parts of Sun Grow Sunshine® Mix #4 (Sun Gro Horticulture, Vancouver, Canada), 1 part of Perlite (Supreme Perlite Company, Portland, OR), and 2 parts of Vermiculite (Therm-O-Rock West Inc., Chandler, AZ) inside 127 mm diameter plastic pots. Plants were fertilized bimonthly with 36-6-6 Miracle-Gro® Lawn Food (The Scotts Company, Marysville, OH). Plants were maintained inside insect-proof cages in a greenhouse with a photoperiod of approximately 12 h and a temperature range of 27 ± 5 °C. They were kept in a separated section of the greenhouse to avoid uncontrolled thrips infestation or MCMV contamination.

MCMV characterization and maintenance. Leaves from corn plants showing typical MCMV mottling symptoms were collected from a corn field at a Pioneer Hi-Bred station at Kekaha (Kauai, Hawaii, USA) in December of 2009. MCMV was transferred to corn seedlings by mechanical inoculation. The virus was characterized by ELISA assays on both the original field infected leaves as well as on the mechanically inoculated seedlings. The plant tissue was screened by ELISA against 6 different corn viruses: *Maize mosaic virus* (MMV) (*Rhabdoviridae*:

Nucleorhabdovirus), MCMV, WSMV, MDMV, SCMV as well as with antibodies that react against the entire Potyvirus genus (AGDIA, Elkhart, IN).

In addition, I sequenced our laboratory isolate of MCMV (MCMV-HI) (4.4 kb) using an approach similar to that described by Stenger and French (2008). Genomic sequences obtained possessed a very high nucleotide similarity, over 97%, to the Nebraska and Kansas strains (results reported in chapter 4). MCMV was transferred monthly to new corn seedlings via mechanical inoculation. Plants were maintained inside insect-proof cages in a greenhouse with a photoperiod of approximately 12 h and a temperature range of 27 ± 5°C.

MCMV purification. Approximately 250 g of MCMV-infected leaves were pulverized in liquid nitrogen and then added to 400 ml of 0.1 M potassium phosphate, pH 7.0, with 1% 2-Mercaptoethanol. The mixture was stirred and filtered through a fine mesh cloth. The extract was then clarified by slowly adding a 0.25 volume of cold chloroform/butanol (1:1, v:v), stirring at 4°C for at least 30 minutes. The solution was centrifuged at 5000 g for 10 min. The aqueous phase was removed and centrifuged at 12000g at 4°C for 10 min. The supernatant was ultra-centrifuged at 270000g, at 4°C, for 90 min. The pellet was resuspended in 4 ml of 0.07 M phosphate buffer and kept at 4°C overnight. The virus preparation went through a second cycle of differential centrifugation (12000g for 10 min, followed by 270000g for 90 min at 4°C), followed by a sucrose gradient purification. Sucrose column was fractionated and fractions contained purified virions were pooled, pelleted by ultracentrifugation, and re-suspended to a final volume of 0.8ml in 0.07 M phosphate buffer. Virus purification was examined by SDS page and spectrophotometer reading.

Genomic RNA isolation and test for the infectivity. RNA was isolated from purified MCMV using TRIzol® LS, according to manufacturer's instructions (Life Technologies, Carlsbad, CA). The RNA was quantified with a NanoDrop and analyzed on a 1% agarose gel for integrity. To test if the genomic RNA was infectious, 3-4 µg of MCMV RNA was mechanically inoculated onto 3-week-old seedlings. Following two weeks. Inoculated plants were tested for MCMV by ELISA.

Thrips transmission of purified MCMV and genomic RNA. I compared the transmission of MCMV following acquisition from purified MCMV virions or MCMV genomic RNA. The genomic RNA concentration (µg/µl) was determined using Beer's law, where the absorbance reading at 260 nm of a purified MCMV preparation is divided by the extinction coefficient of MCMV at 260 nm, calculated by Goldberg and Brakke (1987) at 6.7 cm^2mg^{-1}.

Healthy adult thrips were allowed to artificially feed through a double stretched parafilm membranes containing a 5% sucrose solution with either 30 µg of MCMV RNA in 5% sucrose or purified MCMV virions (4µg/µl) After an acquisition access period (AAP) of 24 h, 30 thrips for each treatment were individually confined to one-week-old corn seedlings inside mesh-ventilated cylindrical, PVC tubes (30 cm tall, 7.5 cm diameter). After an inoculation access period (IAP) of 48 h, the corn seedlings were sprayed with Imidacloprid insecticide (Bayer Advanced, Research Triangle Park, NC) at a concentration of 0.012% and were transferred to a greenhouse for symptom development. After three weeks post-inoculation, the seedlings were additionally tested by ELISA.

3.3 Results

MCMV purification, genomic RNA isolation and infectivity. MCMV was purified by differential centrifugation followed by sucrose gradient separation. The resulting preparation was

visualized on an SDS-PAGE gel, showing a distinct protein having a molecular mass of approximately 25 kDa, as expected for the coat protein of MCMV (Fig. 5). No other proteins were visible on the gel, indicating the virus preparation did not contain contaminants. The genomic RNA of MCMV was isolated from this preparation using Trizol. The integrity of the isolated RNA was visualized on an agarose gel (Fig. 6). All seven corn plants that were challenged with the purified genomic RNA of MCMV showed the characteristic symptoms of infection and was confirmed by ELISA.

Thrips transmission of purified MCMV virions and genomic RNA. Thrips were allowed an acquisition access period of 24 h for transmission of both purified MCMV virions and MCMV genomic RNA. Out of 30 adults tested in 2 replicates, 21 (70%) were able to transmit purified MCMV to the corn seedlings. None of the 33 adult thrips, which were tested for transmission, were able to transmit MCMV by feeding on infectious genomic RNA.

3.4 Discussion

This work demonstrates that the corn thrips is able to acquire purified MCMV virions and to transmit it to the corn seedlings via an artificial feeding assay. As shown by the SDS page, there were no contaminants in the virus preparation. Only MCMV genomic RNA and one capsid protein comprise of the MCMV virions (Gordon et al. 1984). These assays also show that MCMV RNA alone, although infectious, is not transmissible by the corn thrips, suggesting the transmission requires intact viral particles. It is currently unknown whether MCMV may specifically bind to receptors in the thrips vectors.

It is interesting to note that beetle-vectored plant viruses seem to depend upon different strategies for vector transmission than those shown for aphids (Gergerich 2001). For instance, during the feeding process, beetles produce a regurgitant, which is believed to be derived from

secretions of the maxillary and mandibular glands and from the content of the beetle foregut. Regurgitation allows for constant bathing of the mouthparts by plant juices (Fulton et al. 1987). Although it has not been specifically proved for MCMV transmission, it is believed that acquisition and retention of virions by beetles occur through the regurgitant (Fulton et al. 1987) instead of the direct or indirect (in the case of the helper factor strategy) interaction of virions with the arthropod's cuticle, as demonstrated for hemipteran vectors (Ng and Falk 2006). In addition, vector specificity of beetle-transmitted plant viruses depends on the virus particle's interactions with the host plant (Gergerich 2001). Beetle transmitted viruses enter plant tissues through the wound created by beetle chewing and rapidly translocate far from the wounded sites through the plant xylem. Conversely, beetles non-transmissible viruses fail to translocate through the xylem (Gergerich 2001).

As opposed to beetles, thrips have piercing-sucking mouthparts (Nault 1997; Ullman et al. 1992), but they seem to have a very different feeding behavior than several hemipteran insects, such as the aphids. For instance, thrips use stylets to pierce the epidermis and empty the plant cell contents beneath the epidermal layer. The feeding behavior of thrips has been the focus of different studies (Ullman et al. 1992, Stafford et al. 2011). Using electrical penetration graph (EPG) techniques, western flower thrips have been characterized to produce at least three different types of probing: non-ingestion, short-ingestion, and long-ingestion. Short-ingestion and long-ingestion probing are highly destructive to the plant tissues. In contrast, non-ingestion probing leaves the plant cells intact (Stafford et al. 2011). Because before and during non-ingestion probing thrips salivate, it has been suggested that propagative viruses, such as TSWV, may be inoculated to plants during that specific feeding phase (Stafford et al. 2011).

Understanding the corn thrips feeding behavior on corn may be a great advantage to further characterize the transmission biology of MCMV.

The use of localization assays, such as immunofluorescence, may lead to better understanding if MCMV viral particles bind to specific sites in the thrips foreguts or stylets, or if the transmission occurs through a process of ingestion-regurgitation of virions, similar to that described by the beetles vectors of plant viruses. Although several details on the transmission of MCMV are currently not understood, I propose that MCMV can become a valuable model system to dissect and contrast the viral determinants that mediate transmission by arthropod vectors with piercing sucking (corn thrips) and chewing mouthparts (chrysomelid beetles).

Chapter 4. Development of MCMV infectious clones

4.1 Introduction

Plant virus diseases have immense negative impacts on the economy and subsistence of agricultural commodities. Most epidemic plant virus diseases attain high infection rates and spread rapidly when transmitted by arthropod vectors, particularly those belonging to three major orders: Hemiptera, Thysanoptera, and Coleoptera (Nault 1997). The order Hemiptera contains the most important lineages of virus vectors including the aphids, the whiteflies, the leafhoppers, and the planthoppers.

Plant viruses are not transmitted by just any vector species. In general, there exists a high degree of specificity between a virus species and their vectors. For example, the genus Potyvirus (family Potyviridae) lists approximately 70 species, all of which are transmitted by aphids with no evidence that other insect species may transmit them. The same holds true for viral species in the genus Tospovirus (Bunyaviridae), which lists approximately 13 species all of which are transmitted by only thrips. Similarly, beetle transmitted Sobemoviruses (Tombusviridae) are in general not spread by vectors from other arthropod lineages.

The specific relationship between a plant virus and its insect vector(s) is clearly the result of a co-evolutionary process, which have contributed to the adaptation of plant viruses to certain vector lineages and not to others. Molecular genetic studies have revealed that the transmission of plant viruses by vectors is mediated by molecular determinants encoded by viruses. This concept extends also to those viruses that were in the past considered “mechanically” transmitted by their vectors.

Although exceptions do exist, there are few validated reports in literature reporting transmission of the same plant virus by phylogenetically unrelated vector species. However,

Maize chlorotic mottle virus (MCMV) (*Tombusviridae*: *Machlomovirus*) does appear to be one of these exceptions.

Previously, I have shown that the pattern of MCMV transmission by the corn thrips *Frankliniella williamsi* (Thysanoptera) closely resembles that of the same virus by chrysomelid beetles (Coleoptera) (Nault et al. 1978, Jensen 1985). Similar to the western corn rootworm, *Diabrotica virgifera virgifera* LeConte, thrips transmit MCMV soon after acquisition and retain the virus for only a few days with no evidence for a latent period. As in the case of the chrysomelid beetle vectors of MCMV, the corn thrips is able to efficiently transmit the virus as larvae and adults with similar transmission efficiency (Cabanas et al. 2013, Gergerich 2001, Nault et al. 1978). The short retention times, and the lack of virus retention through the thrips development, are also characteristic of MCMV transmission by the corn thrips and the chrysomelid beetles. In summary, my previous research suggests that MCMV is transmitted by corn thrips semi-persistently. In addition, I have demonstrated thrips are able to acquire purified MCMV virions via an artificial feeding assay and to transmit it to the corn seedlings. However, no transmission occurred when only MCMV genomic RNA was supplied in the feeding medium. These results evidence that similarly to the Cucumoviruses such as *Cucumber mosaic virus* (CMV), MCMV likely uses a capsid strategy for vector transmission. For viruses that employ the capsid strategy, changes in the amino acid residues within the ORF encoding for the coat protein could lead to dramatic changes in the transmissibility through their vectors (Ng and Falk 2006).

Infectious clones are important tools to examine the molecular determinants for virus-vector interactions. Previous studies on CMV have shown that inducing mutations on the coat protein sequence resulted in amino acid motifs that altered the physical structure of the coat protein or that caused lack of binding activity to the putative aphid's receptors. The result of these

modifications allowed a CMV isolate, previously aphid non-transmissible, to restore the transmissibility through aphid vectors. The development of infectious clones is the first step to develop a rational approach to study the influence of amino acid residues in MCMV transmission by vectors. As one of the rare viruses transmitted by phylogenetically distant insect species (thrips and beetles), MCMV could serve as model system to examine how residues on the ORF encoding for the coat protein may affect the specificity of transmission.

In this chapter, I first determined the genomic sequence of a laboratory-established isolate of MCMV from Hawaii. After annotating the ORFs, I then attempted to develop infectious clones by producing a full length MCMV DNA sequence carrying the T7 promoter sequence at the 5′-end. The generated PCR product was then used as a template to produce infectious RNA transcripts *in vitro*. Also, I induced mutations to change the amino acid residues found in the Hawaii isolate to that found in US Mainland. This new construct will be used to examine how this point mutation may affect the efficiency of virus transmission by thrips.

4.2 Materials & Methods

Thrips rearing. A colony of corn thrips was established in September 2009 from adults collected from corn ears harvested in a sweet corn field (cv. Golden Cross Bantam (Wetsel Inc., Harrisonburg, VA) on the eastern side of Oahu (Hawaii, USA). Thrips were transferred to potted corn seedlings cv. Golden Cross Bantam inside cylindrical mesh ventilated PVC cages (1016 mm tall, 120 mm in diameter) in a greenhouse with a photoperiod of approximately 12 h and a temperature range of 27 ± 5°C. Newly emerged adults were periodically used for colony maintenance whereas other adults were used for the transmission assays as described hereafter.

MCMV purification. An isolate of MCMV, named MCMV HI, originated form the Kauai Island was transferred to new corn seedlings by mechanical inoculation. Approximately 250 g of

MCMV-infected leaves originated from greenhouse-maintained corn seedlings were pulverized in liquid nitrogen and then added to 400 ml of 0.1 M potassium phosphate, pH 7.0, with 1% 2-Mercaptoethanol. The mixture was stirred and filtered through a fine mesh cloth. The extract was then clarified by slowly adding a 0.25 volume of cold chloroform/butanol (1:1, v:v), stirring at 4°C for at least 30 minutes. The solution was centrifuged at 5000 g for 10 min. The aqueous phase was removed and centrifuged at 12000g at 4°C for 10 min. The supernatant was ultra-centrifuged at 270000g, at 4°C, for 90 min. The pellet was resuspended in 4 ml of 0.07 M phosphate buffer and kept at 4°C overnight. The virus preparation went through a second cycle of differential centrifugation (12000g for 10 min, followed by 270000g for 90 min at 4°C), followed by a sucrose gradient purification. Sucrose column was fractionated and fractions contained purified virions were pooled, pelleted by ultracentrifugation, and re-suspended to a final volume of 0.8ml in 0.07 M phosphate buffer. Virus purification was examined by SDS page and spectrophotometer reading.

RNA Extraction, Reverse Transcription, and long PCR. RNA was isolated from purified MCMV using TRIzol® LS, according to manufacturer's instructions (Life Technologies, Carlsbad, CA). The RNA was quantified with a NanoDrop and analyzed on a 1% agarose gel for integrity. The first strand of cDNA was synthesized using the LongRange 2Step RT-PCR kit (Qiagen, Hilden, Germany) with a MCMV 3′-end specific primer, MCMV_3r (5′-GGGCCGGAAGAGAGGGGCATTACC-3′) designed on the sequence of MCMV Nebraska strain (GenBank Accession EU358605) (Stenger and French 2008). The first strand of cDNA was then amplified using the long range PCR with the following cycling parameters: initial denaturation at 93° for 3 min, 35 cycles of denaturation at 93° for 15 s, annealing at 62° for 30 s, extension at 68° for 4 min and 30s, and a final extension for 5 min at 68°. The primers used were

MCMV_3r and MCMV_5f (5′-AGGTAATCTGCGGCAACAGACCCCAACGCGTTAG-3′), which anchors to the 5′-end of MCMV Nebraska strain genome (Stenger and French 2008). The amplified PCR product was visualized on a 1% agarose gel.

Cloning, sequencing and ORF annotation. The 4.4 kb PCR product was first purified using the MinElute PCR Purification Kit (Qiagen, Venlo, Netherlands), cloned into pCR 4 TOPO vector using the TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA), and transformed into One Shot MAX Efficiency DH5α-T1R Competent Cells (Invitrogen, Carlsbad, CA). Transformed colonies were selected from agar plates and grown overnight in 4 ml Luria Broth (LB) medium with amplicillin. Plasmids were purified using the Plasmid Mini Kit (Qiagen, Venlo, Netherlands). The plasmids were then screened by sequencing using M13 primers and internal primers to obtain the full coverage of the inserts. The consensus sequence of the viral genome of the MCMV HI isolate was determined from sequencing 6 full-length clones. A consensus sequence was assembled and the 6 previously characterized open reading frames of MCMV (Nutter et al. 1989): p111, p50, p31, p32, p7 and p25, which encodes for the coat protein, were annotated.

Production of infectious transcripts. To produce infectious transcripts, I chose a clone that had the consensus sequence for the specific MCMV HI isolate. The primer: MCMV_5f was modified to include a T7 promoter sequence, producing the new primer T7MCMV_5f (5′-TAATACGACTCACTATAAGGTAATCTGCGGCAAC-3′). Using the previously constructed MCMV clone, MCMV was amplified using the long range PCR procedure previously described with primers MCMV_3r and T7MCMV5f to produce full length MCMV DNA sequence carrying the T7 promoter sequence at the 5′-end. This PCR product was then used as a template to produce infectious RNA transcripts using the MEGAscript T7 Kit® (Life Technologies, Carlsbad, CA). Produced RNA was quantified with a NanoDrop and analyzed on a 1% agarose

gel for integrity. The RNA transcripts were then mechanically inoculated onto 3-week-old seedlings that were sprayed with Imidacloprid insecticide (0.012%). The plants were let to develop symptoms for 3 weeks, after which samples were scored for symptoms and tested for presence of the virus by DAS ELISA.

Thrips transmission of MCMV-HI. Adults thrips were transferred to Tashiro cages containing excised MCMV-infected corn leaves from plants infected with RNA transcripts synthesized from the previous experiment. Ninety-nine thrips were allowed to acquire the virus for an acquisition access period (AAP) of 48 h. Thrips were collected and then transferred to two-week-old corn seedlings. Three thrips per caged seedling were used. Following an IAP of 48 h, the seedlings were sprayed with 0.012% Imidacloprid and allowed a three-week period for symptom development. Plants were scored for MCMV infection based upon visual symptoms. Infection was verified by ELISA.

Mutagenesis of MCMVH coat protein. Annotation of the Coat Protein ORF for MCMV Hawaii had a proline residue (CCA) at amino acid site 81, whereas MCMV sequence from the US Mainland carries a serine (TCG) at the same site. Specific primers MCMV_CP81f (5′-TTCCCGGCTGGAACGTCGCCCCGATACATTGGG-3′) and MCMV_CP81r (5′-CCCAATGTATCGGGGCGACGTTCCAGCCGGGAA-3′) were designed to induce a two-base change in the infectious clone pMCMVH CP sequence to recover this serine using GeneArt® Site-Directed Mutagenensis system (Invitrogen). Following mutagenesis and recombination, the mutated MCMV Hawaii plasmids were transformed into One Shot MAX Efficiency DH5α-T1R Competent Cells (Invitrogen). Screening of transformed colonies were done as previously described. The CP ORF of the mutated MCMV Hawaii was sequenced to confirm mutagenesis.

4.3 Results

Sequencing and ORF annotation. I screened 33 clones, 6 of which had inserts of the expected size, 4.4 kb. The consensus sequence obtained by sequencing 6 clones possessed a very high nucleotide similarity, over 97%, to the Nebraska and Kansas strains (GenBank Accessions NC003627 and EU358605). Six ORFs: p111, p50, p31, p32, p7 and p25 were annotated onto the consensus sequence, evidencing a similar genomic organization to MCMV Nebraska and Kansas. However, the ORF encoding for the CP (p25) for MCMV HI had a proline residue (CCA/CCG) at amino acid site 81, whereas the MCMV isolates from the US mainland has a serine (TCG) at the same site (GenBank Accessions NC003627 and EU358605). The presence of the proline residue was confirmed by sequencing the coat protein ORF of five other MCMV isolates collected in the Island of Oahu.

Production of infectious transcripts. Six clones derived from the MCMV HI were screened for infectivity to corn seedlings. Of these six clones, one: pMCMVH, consistently produced RNA transcripts that were infectious to corn plants. RNA transcripts synthesized from pMCMVH had a similar RNA profile to that of natural MCMV RNA (Fig. 7) and displayed similar MCMV symptoms of chlorotic mottling and stunting exhibited by plants challenged by natural MCMV-HI RNA (Fig. 8). Infection was confirmed by ELISA.

Thrips transmission of MCMVH. Out of 99 adults tested in 3 replicates, 15 (15%) were able to transmit pMCMVH by feeding from plants challenged with the *in vitro* synthesized RNA transcripts. Corn thrips transmitted this MCMV with varying rates. In one replicate, none of the thrips transmitted, and in another replicate, only one plant showed MCMV infection (Fig. 9).

Mutagenesis of MCMVH coat protein. A two-base change was induced by site-directed mutagenesis in the CP sequence of the infectious clone pMCMVH to recover the serine found in

mainland isolate of MCMV. The sequence coding for a proline residue in MCMV HI (CCA) was mutated to the mainland sequence (TCG) to produce a new MCMV HI clone having the mainland MCMV coat protein. The new sequence was confirmed by sequencing.

4.4 Discussion

The consensus sequence I obtained of the MCMV HI isolate had over a 97% nucleotide similarity to the Nebraska and Kansas strains (Nutter et al. 1989, Stenger and French 2008). Annotation of the ORFs of the Hawaii isolate showed a similar genomic organization to the mainland isolates. Interestingly, the ORF encoding for the coat protein of MCMV HI carried a single amino acid change at amino acid site 81. While the isolates from the Mainland both contain a serine (TCG) MCMV HI carries a proline residue (CCA for the Kauai island sample and CCG for 5 field samples screened from the Oahu island). Since previous studies have shown that the coat protein is important for virus transmission, this region of the MCMV genome is an ideal target for mutagenesis studies.

Infectious clones have been previously developed to examine the expression strategies and pathogenicity of MCMV (Nutter et al. 1989, Scheets et al. 1993). To begin studying the molecular determinants in MCMV-vector transmission, I developed infectious clones of MCMV from Hawaii (MCMV-HI) using long RT-PCR assays. Of six constructed clones, one consistently produced RNA transcripts that were infectious when inoculated onto corn plants, indicating that the clones produced through long RT PCR are infectious. Plants challenged by this *in vitro* synthesized MCMV-HI RNA transcript displayed similar MCMV symptoms of chlorotic mottling and stunting exhibited by plants challenged by natural MCMV-HI RNA. Thrips could transmit the synthetic virus with varying rates of transmission; however the transmission rates observed tended to be lower than those observed using a genuine MCMV.

It has been postulated that MCMV has been introduced in Hawaii from the trade of MCMV-infected corn seeds form the US Mainland. From an epidemiological perspective, it may be interesting to understand if MCMV is pre-adapted to be transmitted by corn thrips, which has a sub-tropical and tropical distribution and in the US territory is found only in Hawaii. The occurrence of a point mutation in the MCMV HI, which has been observed in multiple samples, may suggest that these may be essential, or important for the efficient transmission of the virus by thrips. In fact, for plant viruses that employ a capsid strategy, the amino acid residues within the coat protein sequence can have a critical influence on the transmissibility by its insect vector (Ng and Falk 2006). In this study it has been possible to use the infectious clone developed to change the proline residue found in MCMV HI to the serine residue that is present in the isolates from the Mainland. The use of these constructs may be used in the future to test how this point mutation may affect the transmission of MCMV by the thrips.

Summary

I have shown that the corn thrips *Frankliniella williamsi* transmit *Maize chlorotic mottle virus* in a semi-persistent manner, similar to the mainland vectors the Chrysomelid beetles. The corn thrips transmitted the virus with no evidence for latent periods and that MCMV persists in larvae and adult thrips for up to 6 days after acquisition, with decreasing transmission rates as time progressed. I did not find evidence of transstadial passage as the adult thrips that acquired the virus as larvae did not transmit MCMV. In real-time RT-PCR assays, I showed that viral load was depleted from the vector's body after thrips had access to healthy plant tissue and that the depletion of viral load was also observed when thrips matured from larvae to adults. I demonstrated that the thrips were able to transmit MCMV after acquisition and inoculation access periods of 3 hours. In addition, I used an artificial feeding assay to feed thrips on purified MCMV or MCMV genomic RNA. Based on the results I achieved, I postulated that MCMV employs a capsid strategy for vector transmission. To further study the molecular determinants in MCMV-vector transmission, I developed infectious clones of MCMV using a long RT-PCR assay. This clone can be used in mutagenesis studies to examine the affects of amino acid changes on transmission of MCMV by the corn thrips. Taken altogether this research suggests that corn thrips transmit MCMV in a semi-persistent manner and that MCMV employs a capsid strategy for vector transmission.

It is currently unknown whether MCMV may specifically bind to receptors in the thrips vectors and it would be interesting to examine the localization of MCMV viral particles in the thrips using localization assays, such as immunofluorescence. Such studies may lead to better understanding of the retention sites in the vector or could determine if the transmission occurs

through a process of ingestion-regurgitation of virions, similar to that described by the beetle vectors of plant viruses.

There are very few examples of plant viruses being spread by phylogenetically unrelated vector species and MCMV is one of these. Although several mechanisms underling MCMV transmission are currently not understood, I propose that MCMV can become a valuable model system to dissect and contrast the molecular determinants that mediate transmission by arthropod vectors with piercing sucking (corn thrips) and chewing mouthparts (chrysomelid beetles). The results presented in this thesis suggest that the approach undertook is effective and could be used in the future to address to these questions, which have great implication in understanding the global epidemic of MCMV.

Figures

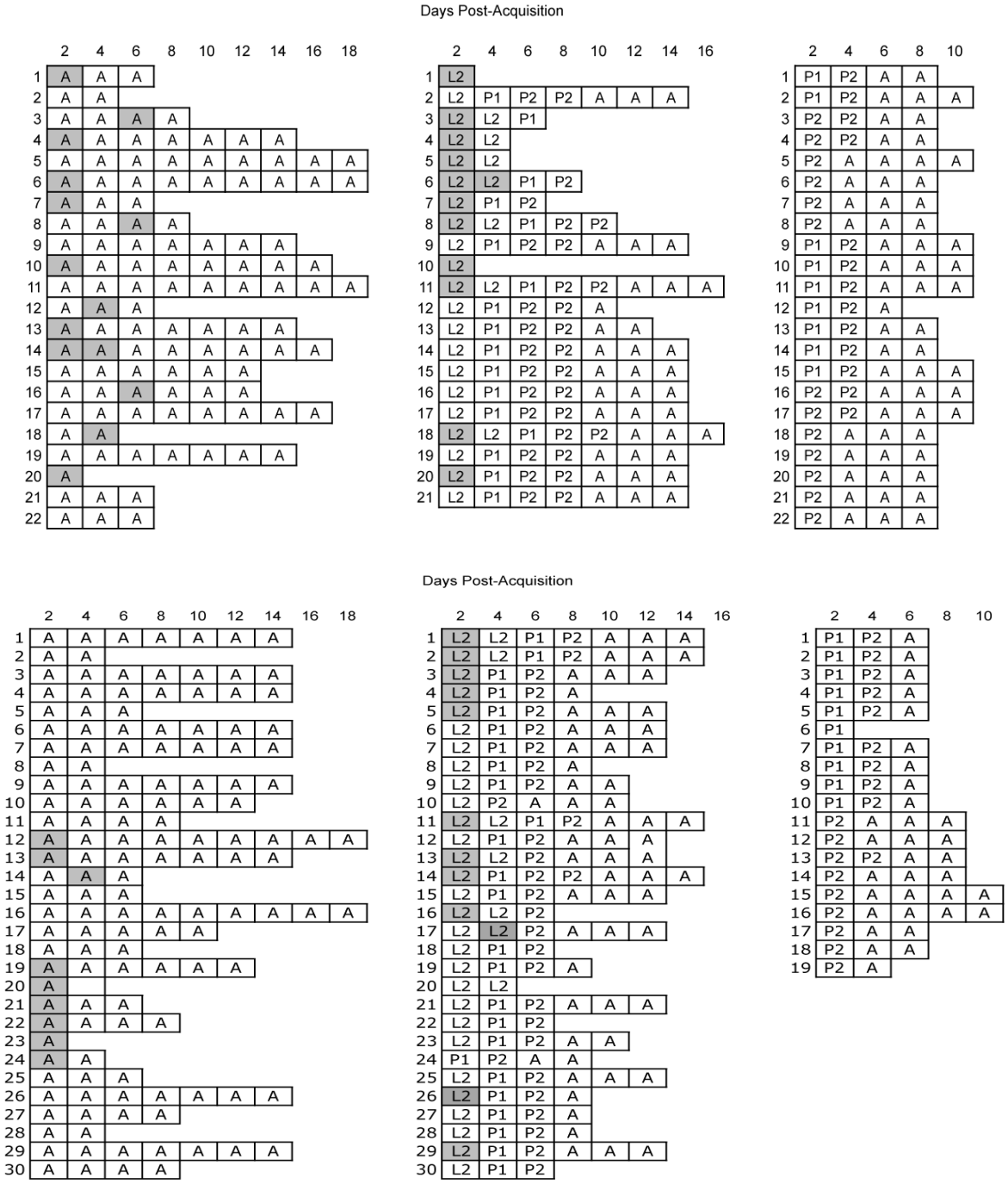

Figure 1. Pattern of *Maize chlorotic mottle virus* (MCMV) transmission by individual corn thrips *Frankliniella williamsi* serially transferred to leaf disks (represented as boxes) every 2 days. Gray and white boxes represent leaf disks that were assayed to be positive and negative for

MCMV by ELISA, respectively. (A) MCMV transmission by thrips that were allowed to acquire the virus as adults by feeding on infected leaves for 2 days. (B) MCMV transmission by thrips that were allowed to acquire the virus as second instar larvae by feeding on infected leaves for 2 days. (C) MCMV transmission by thrips that were transfer to virus-infected leaves as second instar larvae, were allowed to complete their development and were transferred to the leaf disks as pupae. A, adults; L2, second instar larvae; P1, prepupae; P2, pupae.

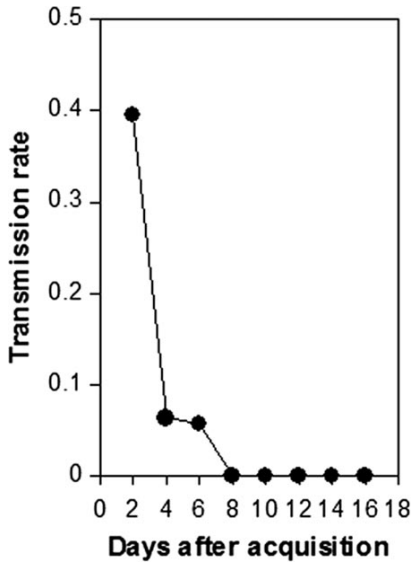

Figure 2. Pattern of *Maize chlorotic mottle virus* (MCMV) transmission by individual corn thrips *Frankliniella williamsi* adults. Thrips were allowed to acquire the virus by feeding on infected leaves for 2 days followed by serial transfers to healthy leaf disks for transmission.

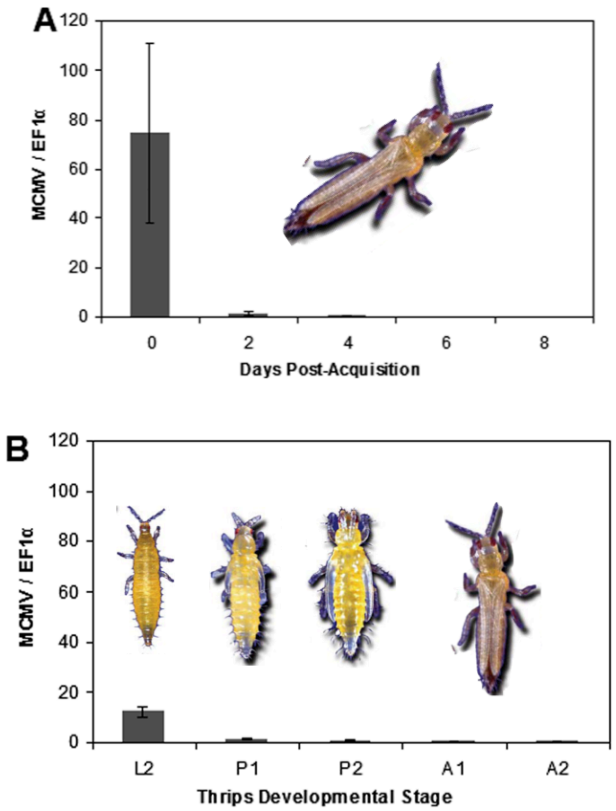

Figure 3. Average concentration ± SEM of *Maize chlorotic mottle virus* (MCMV) relative to the elongation factor-1 alpha (EF-1α) gene of *Frankliniella williamsi*. (A) Average MCMV concentration in adult thrips that were allowed to acquire the virus by feeding on infected leaves for 2 days and that were tested after 0, 2, 4, 6, and 8 days post-acquisition. (B) Average MCMV concentration in thrips that were allowed to acquire the virus for 2 days and that were subsequently tested as second instar larvae (L2) (0 day post-acquisition), prepupae (P1), pupae (P2), 2-day-old adults (A1), 4-day-old adults (A2).

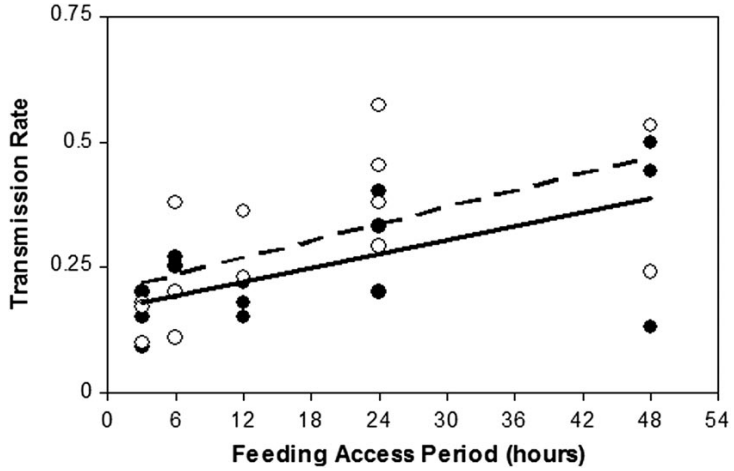

Figure 4. *Maize chlorotic mottle virus* (MCMV) transmission to leaf disks by individual corn thrips *Frankliniella williamsi* adults under different inoculation and acquisition access periods (IAPs and AAPs). Each dot represents the rate of MCMV transmission tested by 15-20 individual thrips. Filled dots represent transmission rates for a 48 h AAP followed by IAPs of: 3, 6, 12, 24 and 48 h. Open dots represent transmission rates for AAPs of: 3, 6, 12, 24 and 48 h followed by an IAP of 48 h. Estimated linear regression for AAP; dashed line: $y = 0.0056x + 0.20$ ($R^2 = 0.33$; $P = 0.026$). Estimated linear regression for IAP; continued line: $y = 0.0046x + 0.16$ ($R^2 = 0.41$; $P = 0.003$).

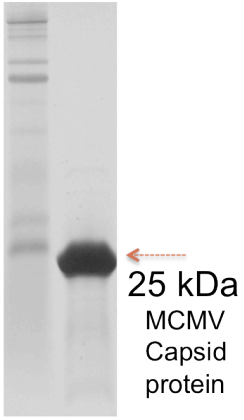

Figure 5. SDS-PAGE of *Maize chlorotic mottle virus* (MCMV) following purification by differential centrifugation and sucrose gradient separation.

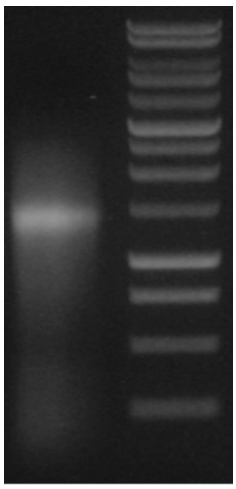

Figure 6. *Maize chlorotic mottle virus* (MCMV) genomic RNA following Trizol isolation.

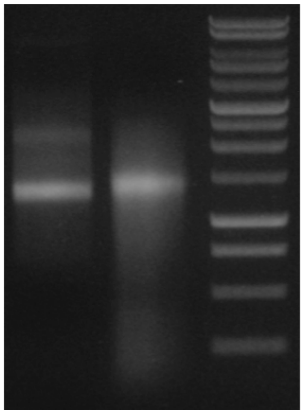

Figure 7. *Maize chlorotic mottle virus* (MCMV) RNA transcripts synthesized from the infectious clone pMCMVH. MCMV-HI RNA transcripts (1) and MCMV-HI RNA (2).

Figure 8. Corn leaf exhibiting chlorotic mottling, the characteristic symptom of *Maize chlorotic mottle virus* (MCMV), following infection by MCMV RNA transcripts.

Replicate	Transmission Rate
1	0/13 (0%)
2	1/7 (14%)
3	4/11 (36%)

Figure 9. Thrips transmission of corn plants infected by RNA transcripts produced by the infectious clone pMCMVH.

```
  1 aggtaatctg cggcaacaga ccccaacgcg ttagacacga ccttacagtt
 51 gactcacgtc ttgcatcctg tgagagctct aacgtgggaa tttgcccctg
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151 ttcaattcac gcattttgga agctgtggtg gccgacgttc tcggaggaac
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4351 taggaaacta gcatcgggcc gcccacgagg gtttctgaac tcaacggagt
4401 agaacggggg ggggtaatgc ccctctcttc cggccc

Figure 10. The genomic consensus sequence of the Hawaii isolate of *Maize chlorotic mottle virus* (MCMV).

Appendix

Dissecting the Mode of Maize Chlorotic Mottle Virus Transmission (Tombusviridae: Machlomovirus) by *Frankliniella williamsi* (Thysanoptera: Thripidae)

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ABSTRACT Maize chlorotic mottle virus (MCMV) (Tombusviridae: Machlomovirus) has been recorded in Hawaii (Kauai Island) since the early 1990s and has since become one of the most widespread corn viruses in the Hawaiian Islands. In the United States Mainland, MCMV has been reported to be transmitted by six different species of chrysomelid beetles, including the western corn rootworm, *Diabrotica virgifera virgifera* LeConte. However, none of these beetle species have been reported in Hawaii where the corn thrips, *Frankliniella williamsi* Hood (Thysanoptera: Thripidae) has been identified to be the main vector. In this study, we developed leaf disk transmission assays and real time reverse transcription-polymerase chain reaction to examine the mode of MCMV transmission by the corn thrips. We showed that thrips transmitted the virus with no evidence for latent periods. Both larvae and adults transmitted the virus for up to 6 d after acquisition, with decreasing rates of transmission as time progressed. There was no evidence that adult thrips that acquired the virus as larvae were competent vectors. Real time reverse-transcription polomerase chain reaction assays showed that viral load was depleted from the vector's body after thrips had access to healthy plant tissue. Depletion of viral load was also observed when thrips matured from larvae to adults. Thrips were able to transmit MCMV after acquisition and inoculation access periods of 3 h. However, transmission efficiency increased with longer acquisition and inoculation access periods. Taken altogether our data suggests that corn thrips transmit MCMV in a semipersistent manner. To our knowledge, this is the first work reporting evidence of a plant virus transmitted semipersistently by thrips.

KEY WORDS leaf disk transmission assay, semipersistent, vector

Maize chlorotic mottle virus (MCMV) (Tombusviridae: *Machlomovirus*) is a plant virus with icosahedral virions of 30 nm in diameter containing a single-stranded positive-sense RNA of ≈4.4 kb (Nutter et al. 1989, Lommel et al. 1991, Stenger and French 2008).

MCMV does not infect dicots but has a wide monocot host range with at least 19 grass species including cultivated ones, such as barley (*Hordeum vulgare* L.), proso millet (*Panicum miliaceum* L.), foxtail millet (*Setaria italica* (L.) Beauvois), and wheat (*Triticum aestivum* L.) (Bockelman et al. 1982). However, to date MCMV causes the most significant economic yield losses to corn (*Zea mays* L.).

MCMV was first described in Peru (Castillo and Hebert 1974) and its current distribution spans several countries in North and South America, including: Argentina (Teyssandier et al. 1983), Brazil, Mexico (Gordon et al. 1984), and the United States (Niblett and Claflin 1978, Uyemoto et al. 1980, Jiang et al. 1992). Recently, the virus has also been recorded in some Asian countries, such as China (Xie et al. 2011) and Thailand (Scheets 2008). In the United States, MCMV is present in Kansas (Niblett and Claflin 1978), Nebraska (Doupnik 1979), Texas (Nyvall 1999), and Hawaii (Jiang et al. 1992). Because MCMV is transmitted at low rates (0.008–0.04%) through the corn seeds (Jensen et al. 1991), it has been suggested the trades of MCMV-infected seeds has contributed to the wide distribution of this virus (Jensen et al. 1991, Scheets 2008).

Corn plants infected by MCMV show symptoms of chlorotic mottling on leaves, and stunting in growth. It has been reported that MCMV infection can reduce crop yield by 10–15% (Castillo and Hebert 1974, Loayza 1976, Nault et al. 1981). When MCMV co-infects corn plants with other corn viruses from the family *Potyviridae*, such as maize dwarf mosaic virus (MDMV) (genus: *Potyvirus*), sugar cane mosaic virus (SCMV) (genus: *Potyvirus*), or wheat streak mosaic virus (WSMV) (genus: *Tritimovirus*), their synergistic effect causes a more severe disease called Corn Lethal Necrosis (CLN) (Uyemoto et al. 1980, Goldberg and Brakke 1987). Symptoms of CLN include leaf necrosis,

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severe plant stunting, and premature death. CLN-infected plants have average MCMV concentrations 3–11 times higher than singularly infected MCMV plants (Goldberg and Brakke 1987, Scheets 1998). It has been reported that CLN disease can reduce crop yield by up to 90% (Niblett and Claflin 1978, Uyemoto et al. 1980).

Nault and colleagues (1978) showed that six species of Chrysomelid beetles are vectors of MCMV. These include: the western corn rootworm *Diabrotica virgifera virgifera* LeConte, the northern corn rootworm *D. barberi* Smith & Lawrence, the southern corn rootworm *D. undecimpunctata howardi* Barber, the corn flea beetle *Chaetocnema pulicaria* Melsheimer, the flea beetle *Systena frontalis* (F.), and the cereal leaf beetle *Oulema melanopus* (L.). Eight other potential vector species screened by Nault and colleagues (1978), including the Japanese beetle *Popillia japonica* Newman, the black cutworm *Agrotis ipsilon* (Hufnagel), the bird cherry-oat aphid *Rhopalosiphum padi* L., the green peach aphid *Myzus persicae* (Sulzer), the greenbug *Schizaphis graminum* (Rodani), the whitefly *Trialeurodes vaporariorum* Westwood, the corn planthopper *Peregrinus maidis* (Ashmead), and the blackfaced leafhopper *Graminella nigrifrons* (Forbes), failed to transmit the virus. The beetle species *D. viridula* (F.) and *D. decempunctata* (Latreille) were also identified as vectors of MCMV in Peru (Castillo 1983).

Transmission of MCMV by chrysomelid beetles resembles that of many other plant viruses transmitted by beetles (Gergerich 2001). For instance, Nault et al. (1978) found that MCMV was transmitted immediately after acquisition and persisted for up to 6 d in the western corn rootworm and in the cereal leaf beetle. In addition, there was no evidence of transstadial passage because larvae of the cereal leaf beetle lost the ability to transmit the virus after adult eclosion. Larvae of the cereal leaf beetle were more efficient vectors of MCMV than their adult counterparts. Jensen (1985) performed additional transmission experiments by using three corn rootworm species: the southern, the northern, and the western corn rootworm. The author did not find evidence of a latent period nor of transovarial passage. Furthermore, he found that MCMV had to be acquired through insect feeding, and that surface contamination alone was not enough to support transmission. Larvae were shown to effectively transmit MCMV, which was recovered in some trace amounts, in the insect's gut and hemolymph (Jensen 1985).

Because none of the chrysomelid species vectors of MCMV occurred across the Hawaiian Islands (Nishida 2002), Jiang and colleagues (1992) conducted an additional study aimed at identifying potential arthropod vectors of MCMV in cornfields cultivated in the island of Kauai (Hawaii). Of six arthropod species tested: *P. maidis*, *Sardia pluto* (Kirkaldy), the southern garden leafhopper *Empoasca solana* DeLong, the Chinese rose beetle *Adoretus sinicus* Burmeister, mites of the genus *Tetranychus* spp. and the corn thrips *Frankliniella williamsi* Hood, the authors found that the sole species capable of transmitting MCMV was the corn thrips, *F. williamsi* (Jiang et al. 1992).

To date, it is not known through which mode the corn thrips may transmit MCMV. Currently, thrips are known to transmit plant viruses belonging to different genera: *Tospovirus*, *Ilarvirus*, *Carmovirus*, and *Sobemovirus* (Jones 2005, Whitfield et al. 2005). These viruses can be transmitted by thrips in different ways. For instance, tomato spotted wilt virus (TSWV) (*Bunyaviridae: Tospovirus*) is transmitted by the western flower thrips, *Frankliniella occidentalis* (Pergande) and other thrips species in a propagative-persistent manner (Wijkamp et al. 1996, Whitfield et al. 2005). TSWV is generally acquired by the first instar larvae and is transmitted after adult eclosion (Wijkamp and Peters 1993, Ullman et al. 1992). The replication of the virus within the thrips body ensures the persistence of transmission over long periods of time (Whitfield et al. 2005). Other viruses, like tobacco streak virus (TSV) (*Bromoviridae: Ilarvirus*), pelargonium flower break virus (PFBV) (*Tombusviridae: Carmovirus*), or sowbane mosaic virus (SoMV) (*Sobemovirus*) are transmitted by the thrips as a sort of "mechanical accident" (Krczal et al. 1995, Hardy and Teakle 1992). All of these viruses seem to be pollen-borne (Jones 2005). For PFBV and SoMV, it has been shown that thrips may transport virus-infected pollen from infected plants to healthy ones and facilitate inoculation through feeding wounds. Thrips may also facilitate the transmission of virus by introducing through feeding the infected pollen that covers the leaf surface, as demonstrated for TSV (Reddy et al. 2002, Jones 2005). None of these viruses seem to directly interact with the thrips vectors.

In this work we examined the mode of MCMV transmission by the corn thrips. We tested the competence of virus transmission by larvae and adults, the persistence of transmission, and the possible retention through thrips development. We adopted a real-time reverse transcription polymerase chain reaction (RT-PCR) assay to quantify the change in viral load in thrips over time and throughout their development. Finally, we tested the variation of MCMV acquisition and inoculation efficiency over time.

Materials and Methods

Thrips Rearing and Production of Corn Seedlings. A colony of corn thrips was established in September 2009 from adults collected from corn ears harvested in a sweet corn field (Golden Cross Bantam, Wetsel Inc., Harrisonburg, VA) on the eastern side of Oahu (Hawaii).

Thrips were transferred to potted corn seedlings Golden Cross Bantam inside cylindrical mesh ventilated PVC cages (1,016 mm tall, 120 mm in diameter) in a greenhouse with a photoperiod of ≈12 h and a temperature range of 27 ± 5°C. To produce coetaneous insects, ≈40–50 adults were transferred to caged 3-wk-old corn seedlings and were allowed to lay eggs for 1 wk. Adults were thereafter removed from cages and the eggs laid were allowed to develop into adults, which appeared in approximately 3 to 4 wk. Newly emerged adults were periodically used for colony

maintenance whereas other adults or larvae were used for the transmission assays as described hereafter. Leaf samples from the corn seedlings used for thrips rearing were periodically tested by double antibody sandwich enzyme-linked immunosorbant assay (DAS-ELISA) for MCMV to ensure the insect colony was not infected.

Corn seedlings of Golden Cross Bantam used to rear thrips and for the transmission assays were sown in a soil mix made up of three parts of Sun Grow Sunshine Mix #4 (Sun Gro Horticulture, Vancouver, Canada), one part of Perlite (Supreme Perlite Company, Portland, OR), and two parts of Vermiculite (Therm-O-Rock West Inc., Chandler, AZ) inside 127 mm diameter plastic pots. Plants were fertilized bimonthly with 36–6-6 Miracle-Gro Lawn Food (The Scotts Company, Marysville, OH). Plants were maintained inside insect-proof cages in a greenhouse with a photoperiod of ≈12 h and a temperature range of 27 ± 5°C. They were kept in a separated section of the greenhouse to avoid uncontrolled thrips infestation or MCMV contamination.

MCMV Characterization and Maintenance. Leaves from corn plants showing typical MCMV mottling symptoms were collected from a corn field at a Pioneer Hi-Bred station at Kekaha (Kauai, HI) in December of 2009. MCMV was transferred to corn seedlings by mechanical inoculation. The virus was characterized by ELISA assays on both the original field infected leaves as well as on the mechanically inoculated seedlings. We screened the plant tissue by ELISA against six different corn viruses: maize mosaic virus (MMV) (*Rhabdoviridae: Nucleorhabdovirus*), MCMV, WSMV, MDMV, SCMV as well as with antibodies that react against the entire Potyvirus genus (AGDIA, Elkhart, IN).

In addition, we sequenced our laboratory isolate of MCMV (MCMV-HI) (4.4 kb) using an approach similar to that described by Stenger and French (2008). Genomic sequences obtained possessed a very high nucleotide similarity, over 98%, to the Nebraska and Kansas strains (unpublished). MCMV was transferred monthly to new corn seedlings via mechanical inoculation. Plants were maintained inside insect-proof cages in a greenhouse with a photoperiod of ≈12 h and a temperature range of 27 ± 5°C.

MCMV Transmission to Leaf Disks and to Corn Seedlings. We compared in preliminary experiments the transmission of MCMV to corn seedlings or to leaf disks. Healthy adults, starved for 2 h, were transferred into Tashiro cages (Tashiro 1967) containing a portion of excised MCMV-infected corn leaf. After an acquisition access period (AAP) of 48 h, 20 thrips were individually confined to 1-wk-old corn seedlings inside mesh-ventilated cylindrical, PVC tubes (30 cm tall, 7.5 cm diameter). After an inoculation access period (IAP) of 48 h, the corn seedlings were sprayed with Imidacloprid insecticide (Bayer Advanced, Research Triangle Park, NC) at a concentration of 0.012% and were transferred to a greenhouse for symptom development. Alternatively, MCMV transmission was tested using a modified leaf disk assay (Wijkamp and Peters 1993). Leaf disk chambers were created from 15-ml Falcon tubes by transversely cutting tubes 15 mm below the lid. Leaf disks, 14 mm in diameter, were excised from healthy corn leaf midribs. One milliliter of melted 1% agar solution was poured into the cap and a leaf disk was placed on the surface before the agar solidified. After an AAP of 48 h, 20 thrips were individually confined to leaf disk chambers. Chambers were sealed with stretched Parafilm and pierced on the top with a pin to allow ventilation. At the end of an IAP of 48 h, thrips were removed from the chambers and transferred into 1.5 ml tubes containing 70% ethanol. The leaf disks were incubated in their chambers at 25 ± 2°C for another 4 d to allow the virus to replicate. Leaf disks were thereafter removed from the chamber, cleaned from the agar by washing under tap water, and processed for ELISA to test for the presence of MCMV as described below. Five uninfected thrips collected from rearing colonies were used as controls.

Transmission to either leaf disks or seedlings were replicated three times. For statistical analysis, the proportion of MCMV-infected leaf disks or seedlings were transformed by arcsine root square and compared with a *t*-test using the statistical software SigmaStat Version 3.5 (San Jose, CA).

Persistence. Persistence experiments used the previously described leaf disk assay with some modification. Healthy adults, starved for 2 h, were transferred to Tashiro cages containing excised MCMV-infected corn leaves. After an AAP of 48 h, thrips were starved for 2 h and individually transferred to healthy corn leaf disks for an IAP of 48 h. Each thrips was thereafter serially transferred every 48 h to new leaf disks. Experiments lasted for the entire lifespan of the thrips. Leaf disks were handled as previously described. DAS-ELISA tests were performed on the leaf disks to test for the presence of MCMV. Persistence experiments were replicated twice.

Virus Retention Through Thrips Development. To examine the retention of MCMV through thrips development, second instar larvae were collected from rearing cages and were starved for 2 h before being transferred to Tashiro cages, where they were allowed to feed on excised MCMV-infected corn leaves for an AAP of 48 h or until reaching their pupal stage. Pupae were then individually transferred to healthy corn leaf disks for a period of 48 h, thrips were serially transferred for two to three passages to healthy leaf disks with an IAP of 48 h each. DAS-ELISA tests were performed on the leaf disks to test for the presence of MCMV. Real-time RT-PCR described below was used to quantify viral load in thrips.

Transmission Efficiency. Adults starved for 2 h were transferred to Tashiro cages containing excised MCMV-infected corn leaves. Five batches of 20 thrips were allowed to feed for AAPs of 3, 6, 12, 24, or 48 h. Insects were collected from the cages at the end of each assigned acquisition period, starved for another 2 h, then individually transferred to healthy corn leaf disks for an IAP of 48 h. At the end of the IAP, thrips were removed and transferred into 1.5 ml tubes con-

taining 70% ethanol, whereas the leaf disks were handled as previously described to allow for virus replication. A similar procedure was used for another series of transmission experiments where batches of 20 thrips were allowed to acquire MCMV by feeding on infected leaves for AAP of 48 h followed by IAPs of 3, 6, 12, 24, or 48 h. We conducted additional tests to examine the possibility that thrips may transmit MCMV similarly of aphid-transmitted nonpersistent viruses (Ng and Falk 2006). For these assays we allowed 2 h starved adult thrips to feed on MCMV-infected corn leaves for an AAP of 10 min, then batches of three insects were transferred to healthy corn leaf disks for an IAP of 1 h. These experiments were replicated three times. DAS-ELISA tests were performed on the leaf disks to detect for the presence of MCMV. Transmission rates were plotted against time and were fitted to linear regressions approximated using least squares approach of the statistical software SigmaStat Version 3.5.

ELISA. Leaf disks used in transmission assays were tested for MCMV by DAS-ELISA. Leaf extracts were prepared by grinding each leaf disk with pestles in a 1.5 ml tube containing 300 μl of extraction buffer (10.3 mM Na_2SO_3, 2% Polyvinylpyrrolidone, 3.1 mM NaN_3, 0.2% powdered chicken egg albumin, and 2% Tween 20, pH 7.4). ELISA was performed according to manufacturer's instructions (AGDIA). After color development, a reading of absorbance values was taken at $A_{405\ nm}$ using a microplate reader (model 680, Bio-Rad, Hercules, CA). Positive readings were determined as values greater than twice the average reading of the negative controls. MCMV-infected and healthy corn leaf samples were used as positive and negative controls, respectively. ELISA tests were used to confirm for the presence of MCMV in mechanically inoculated corn tissues used for the transmission tests.

RNA Extraction and Reverse Transcription. RNA was isolated from leaf disks excised from infected or from healthy seedlings as well as from thrips produced for the persistence and the retention through thrips development experiments using an RNeasy Mini Kit (Qiagen, Valencia, CA). Leaf disks or groups of three thrips were ground in a 1.5 ml tube containing 350 μl buffer RLT. Samples were vortexed for 5 min and then centrifuged in QIAshredder columns (Qiagen) for 2 min, high speed. RNA extraction was performed according to manufacturer's instructions (Qiagen). Samples were then reverse transcribed using a QuantiTect Reverse Transcription Kit (Qiagen).

Real-Time RT-PCR. We adopted a real-time RT-PCR assay to quantify MCMV in thrips bodies using a similar procedure adopted by Rotenberg et al. (2009) to quantify TSWV within the western flower thrips. We performed a relative quantification targeting the MCMV open reading frame encoding for the coat protein, and as a reference gene, the thrips elongation factor-1 alpha (EF-1α). The universal EF-1α gene primers Manto (5′-GGAACBTCWCAGGCTGAYTGTGC-3′) and Phasma (5′-GGCGCRAADGTNACNACCATDCCRGG-3′) described by Djernaes and Damgaard (2006) were used to obtain a 532 bp from the EF-1α gene of the corn thrips. The amplicon obtained was sequenced from the 5′ and 3′ ends and used with the software Primer3 (v. 0.4.0) to design a primer pair specific to the corn thrips EF-1α gene, EF_fwf (5′-TCTCCAAGAACGGACAGACC-3′) and EF_Fwr (5′-GATCAGCTGCTTCACACCAA-3′). Nucleotide sequences generated from MCMV-HI were used to design the primer pair MCMV_CPf (5′-TTAACTCTGTGCGCATCACC-3′) and MCMV_CPr (5′-ATCCGCTAGTGGTGTCTGCT-3′). The specificity of the primer pairs were tested in PCR assays and were confirmed by sequencing the amplicons obtained as well as in melting curve analysis.

Real-time RT-PCR reactions were prepared with a master mix containing 10 μl of SYBR Green (Qiagen), 1 μl of 10 μM of each primer, and 2.5 μl of cDNA in a final volume of 20 μl. Samples were amplified in duplicates using a Rotor-Gene 6000 Thermal Cycler (Corbett Research, San Francisco, CA). The relative amount of MCMV cDNA was normalized to the amount of EF-1α cDNA using the equation described by Pfaffl (2001): $E^{Ct(EF\text{-}1\alpha)}/E^{Ct(MCMV)}$; where E = PCR efficiency of a given primer pair, and Ct = cycle threshold, which was automatically calculated by the PCR machine's software (Rotor-Gene 6000 Series Software Version 1.7, Corbett Research). Efficiency values were calculated by using the formula: $E = 10^{(-1/slope)}$. The slopes of the curves were determined empirically by four-fold serial dilutions. Because E = 2.00 was achieved for both MCMV and EF-1α primer pairs, we adopted a simplified equation: $two^{Ct(EF\text{-}1\alpha\ -\ MCMV)}$ (Pfaffl 2001) to calculate the relative amount of MCMV in each sample. To analyze data statistically, the relative concentrations of MCMV within thrips were replicated five times, each consisting of three-pooled thrips. Data were examined using a Kruskal–Wallis test. Pairwise comparisons were tested for differences adopting a Mann–Whitney *U* test. Both statistical tests were performed with the software SigmaStat Version 3.5.

Results

Transmission of MCMV to Seedlings and Leaf Disks. No statistical differences were observed in the rates of MCMV transmission by individual thrips using corn seedlings or leaf disks, which averaged at 0.37 ± 0.08 (SE) and at 0.39 ± 0.08 (SE), respectively (t = 0.17; df = 4; P = 0.87). Therefore, leaf disks were used as a routine technique for the subsequent transmission assays.

Persistence. Adults were allowed to feed for 48 h on MCMV-infected leaves followed by serial passages to healthy leaf disks on which individual thrips fed for 48 h IAPs. Out of 52 thrips tested in two replicates, 22 (42%) were able to transmit MCMV (Fig. 1A; Supp. Fig. S1A [online only]). When virus transmission was examined over time, we observed a decrease in the rate of transmission (Fig. 2). The virus was not transmitted by thrips beyond 6 d postacquisition (Fig. 1A; Supp. Fig. S1A [online only]). Of the 22 transmitters, 21 (95%) inoculated MCMV to only one leaf disk, and

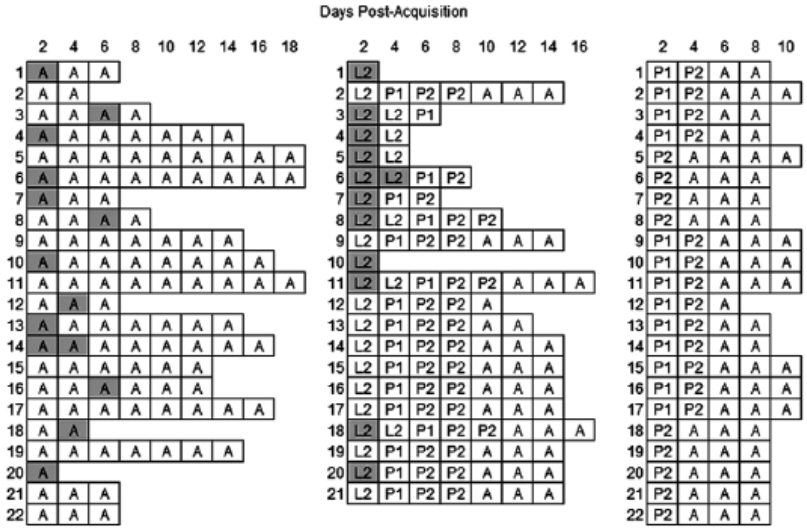

Fig. 1. Pattern of maize chlorotic mottle virus (MCMV) transmission by individual corn thrips *Frankliniella williamsi* serially transferred to leaf disks (represented as boxes) every 2 d. Gray and white boxes represent leaf disks that were assayed to be positive and negative for MCMV by ELISA, respectively. (A) MCMV transmission by thrips that were allowed to acquire the virus as adults by feeding on infected leaves for 2 d. (B) MCMV transmission by thrips that were allowed to acquire the virus as second instar larvae by feeding on infected leaves for 2 d. (C) MCMV transmission by thrips that were transfer to virus-infected leaves as second instar larvae, were allowed to complete their development and were transferred to the leaf disks as pupae. A, adults; L2, second instar larvae; P1, prepupae; P2, pupae.

16 out of 22 (73%) thrips transmitted MCMV to the first leaf disk used in the serial passages.

Real time RT-PCR showed a decrease in the average concentration of MCMV in the thrips (Fig. 3A). Significant differences were observed when the Kruskal–Wallis test was applied ($H = 16.45$; df = 4; $P = 0.002$). The titer of MCMV relative to the thrips gene EF-1α peaked at the end of the 2 d AAP (reported in Fig. 3A as day 0) at averages of 74.3 to only 1.3 after thrips had access to healthy leaves for an additional 2 d ($P = 0.016$; Mann–Whitney test) thus evidencing depletion of the virus from the thrips.

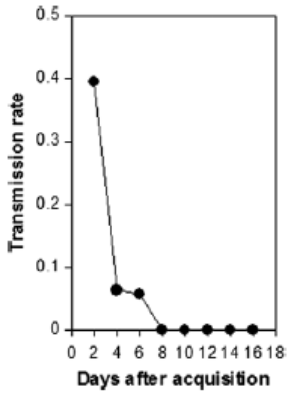

Fig. 2. Pattern of maize chlorotic mottle virus (MCMV) transmission by individual corn thrips *Frankliniella williamsi* adults. Thrips were allowed to acquire the virus by feeding on infected leaves for 2 d followed by serial transfers to healthy leaf disks for transmission.

Virus Retention Through Thrips Development. Out of 54 s instar larvae tested in two replicates, 24 (44%) were able to transmit MCMV (Fig. 1B; Supp. Fig. S1B [online only]). However, the pupae and the emerging adults that developed from those larvae were no longer able to transmit the virus (Fig. 1B; Supp. Fig. S1B [online only]). Similarly, when 41 larvae, tested in two replicates, were allowed to complete their development on MCMV-infected leaves, the pupae and adults that were subsequently transferred to the leaf disks were unable to transmit the virus (Fig. 1C; Supp. Fig. S1C [online only]). Real time RT-PCR showed a decrease in the average concentration of MCMV in the thrips (Fig. 3B). Significant differences were detected when the Kruskal–Wallis test was applied ($H = 12.95$; df = 4; $P = 0.012$). The relative titer of MCMV in the larvae was 10–30 times larger than what we observed into the pupae or emerging adults ($P < 0.05$; Mann–Whitney test), indicating a rapid depletion of the virus during the thrips development. As shown by contrasting Fig. 3A and B, viral load into the adults was six times larger than what observed into the larvae ($P < 0.05$; Mann–Whitney test).

Transmission Efficiency. None of the 60 adult thrips, which were tested for transmission in batches of 3, were able to transmit MCMV by feeding on virus-infected corn leaves for an AAP of 10 min followed by feeding on healthy corn leaf disks for an IAP of 1 h, suggesting MCMV is not vectored with a non-persistent transmission mode. However, individual adult thrips were able to transmit MCMV after AAP and IAP of 3 h (Fig. 4). In addition, the transmission

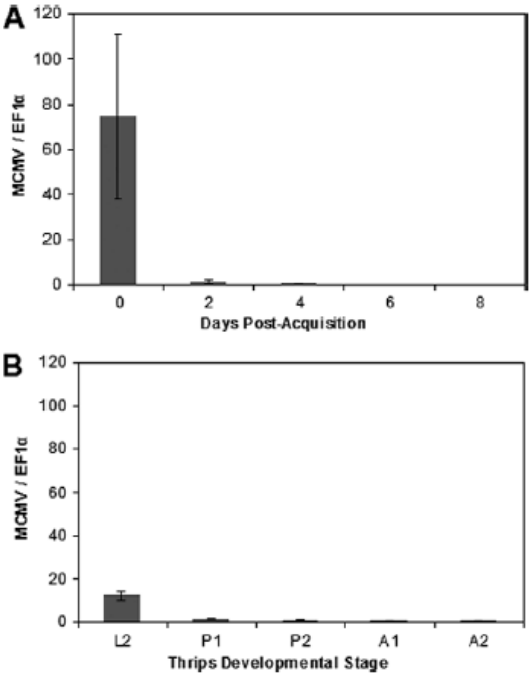

Fig. 3. Average concentration ± SEM of maize chlorotic mottle virus (MCMV) relative to the elongation factor-1 alpha (EF-1α) gene of *Frankliniella williamsi*. (A) Average MCMV concentration in adult thrips that were allowed to acquire the virus by feeding on infected leaves for 2 d and that were tested after 0, 2, 4, 6, and 8 d postacquisition. (B) Average MCMV concentration in thrips that were allowed to acquire the virus for 2 d and that were subsequently tested as second instar larvae (L2) (0 d postacquisition), prepupae (P1), pupae (P2), 2-d-old adults (A1), and 4-d-old adults (A2).

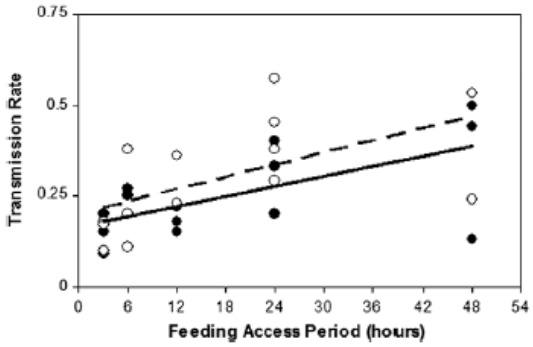

Fig. 4. Maize chlorotic mottle virus (MCMV) transmission to leaf disks by individual corn thrips *Frankliniella williamsi* adults under different inoculation and acquisition access periods (IAPs and AAPs). Each dot represents the rate of MCMV transmission tested by 15–20 individual thrips. Filled dots represent transmission rates for a 48 h AAP followed by IAPs of: 3, 6, 12, 24, and 48 h. Open dots represent transmission rates for AAPs of: 3, 6, 12, 24, and 48 h followed by an IAP of 48 h. Estimated linear regression for AAP; dashed line: $y = 0.0056x + 0.20$ ($R^2 = 0.33$; $P = 0.026$). Estimated linear regression for IAP; continued line: $y = 0.0046x + 0.16$ ($R^2 = 0.41$; $P = 0.003$).

efficiency increased when thrips had longer AAPs and IAPs after linear regressions over time (Fig. 4).

Discussion

To our understanding, this is the first study reporting experimental evidence on the mode of MCMV transmission by the corn thrips. For most of our transmission assays we elected to condition insects to a fasting period because we noticed thrips spent lots of time walking into the chambers rather than attempt feeding on the leaf disks. We also elected to use IAPs of 2 d because this was shown in our preliminary transmission assays to support high rates of MCMV transmission. Furthermore, transmission and inoculation access periods ranging from 1 to 4 d have been previously adopted to examine the transmission of MCMV by the chrysomelid beetles (Nault et al. 1978, Jensen 1985). In addition, we tested MCMV transmission under acquisition and inoculation times required by aphid-transmitted nonpersistent viruses, however we failed to obtain evidence of virus transmission under these experimental conditions.

TSWV, which propagates within the thrips vectors, is generally acquired by the larvae and then is persistently transmitted after adult eclosion (Wijkamp and Peters 1993, Ullman et al. 1992). The pattern of TSWV transmission contrasts with the pattern of transmission observed with MCMV. In fact, the corn thrips larvae lose the virus soon after feeding on healthy plant tissues. Even when the second instar larvae are allowed completing their development on MCMV-infected leaves, neither the pupae nor the emerging adults are competent in transmitting the virus. These results are important under an epidemiological point of view as they suggest that MCMV likely spread through the vector activity of adult thrips, which are responsible for both phases of virus acquisition and inoculation. Because larvae have a limited dispersal activity, they likely have a limited role in vectoring MCMV even when they develop from virus-infected plants.

The pattern of MCMV transmission by the corn thrips closely resembles those for the same virus by Chrysomelid beetles (Nault et al. 1978, Jensen 1985). Similar to the western corn rootworm, thrips transmit MCMV soon after acquisition and retain the virus for only a few days with no evidence for a latent period. As in the case of the chrysomelid beetle vectors of MCMV, the corn thrips is able to efficiently transmit the virus as larvae and adults with similar transmission efficiency (Gergerich 2001, Nault et al. 1978). The short retention times, and the lack of virus retention through the thrips development, are also the characteristics of MCMV transmission by the corn thrips and the Chrysomelid beetles.

Quantification of MCMV by real time-RT PCR showed a depletion of the viral load after thrips were transferred to feed on the healthy corn tissues. Sim-

ilarly, thrips lost the entire viral load through their development from larvae to adults. Although both larvae and adults were competent in vectoring MCMV, the viral load in adults was determined to be at least six times greater than what observed in larvae. Altogether, the data obtained by real-time RT-PCR is consistent with the results from transmission assays. Collectively, these results suggest that MCMV is transmitted semipersistently by the corn thrips. Semipersistent viruses generally persist in their vectors for a few days, they are not retained after the molting, and have no detectable latent period (time between acquisition and inoculation) in their vectors (Ng and Falk 2006). To our knowledge, this is the first work reporting evidence of a plant virus transmitted semipersistently by thrips.

To date, is unclear why the MCMV is not retained through the thrips development; both thrips molting and time passing can contribute to the observed depletion of virus into the insect. Future additional assays that consider shorter feeding time may shed lights to the reasons underling the lack or virus retention. In general, hemipteran vectors retain semipersistent viruses in the mouthpart or anterior midgut (Chen et al. 2011, Ng and Falk 2006, Uzest et al. 2011). Virus binding is mediated either directly by the virus coat protein (capsid strategy) or indirectly though virus-derived nonstructural proteins (helper factor strategy) (Ng and Falk 2006). Conversely, beetle-vectored plant viruses seem to depend upon different strategies for vector transmission (Gergerich 2001). For instance, during the feeding process, beetles produce a regurgitant, which is believed to be derived from secretions of the maxillary and mandibular glands and from the content of the beetle foregut. Regurgitation allows for constant bathing of the mouthparts by plant juices (Fulton et al. 1987). Although it has not been specifically proved for MCMV transmission, it is believed that acquisition and retention of virions by beetles occur through the regurgitant (Fulton et al. 1987) instead of the direct or indirect (in the case of the helper factor strategy) interaction of virions with the arthropod's cuticle, as demonstrated for hemipteran vectors (Ng and Falk 2006). In addition, vector specificity of beetle-transmitted plant viruses depends on the virus particle's interactions with the host plant (Gergerich 2001). Beetle transmitted viruses enter plant tissues through the wound created by beetle chewing and rapidly translocate far from the wounded sites through the plant xylem. Conversely, beetles nontransmissible viruses fail to translocate through the xylem (Gergerich 2001).

As opposed to beetles, thrips have piercing-sucking mouthparts (Nault 1997, Ullman et al. 1992). Thrips use stylets to pierce the epidermis and empty the plant cell contents beneath the epidermal layer. The feeding behavior of thrips has been the focus of different studies (Ullman et al. 1992, Stafford et al. 2011). Using electrical penetration graph (EPG) techniques, western flower thrips have been characterized to produce at least three different types of probing: noningestion, short-ingestion, and long-ingestion. Short-ingestion and long-ingestion probing are highly destructive to the plant tissues. In contrast, noningestion probing leaves the plant cells intact (Stafford et al. 2011). Because before and during noningestion probing thrips salivate, it has been suggested that propagative viruses, such as TSWV, may be inoculated to plants during that specific feeding phase (Stafford et al. 2011). Understanding the corn thrips feeding behavior on corn may be a great advantage to further characterize the transmission biology of MCMV.

In general, plant viruses belonging to the same genus are transmitted by specific vector taxa (Nault 1997, Ng and Falk 2006). For example, the plant virus genus *Potyvirus* (family *Potyviridae*) is comprised of at least 118 species, all of which are transmitted by aphids (Raccah et al. 2001, Ng and Falk 2006). The same holds true for the 14 virus species comprising the genus *Tospovirus* (*Bunyaviridae*), all of which are transmitted by thrips (Whitfield et al. 2005). In most cases, plant viruses that are transmitted by leaf-feeding beetles are not transmissible by other arthropod vectors (Gergerich 2001). These trends of association are commonly observed across several plant virus genera (Nault 1997) and are likely the result of a process of co-evolution. In addition, several studies have been revealing that vector-virus specificity is regulated at the molecular and cellular level, and the molecular determinants involved in virus-vector recognition are often specific (Gergerich 2001, Hogenhout et al. 2008, Ng and Falk 2006). Therefore, the transmission of MCMV by both corn thrips (order Thysanoptera) and Chrysomelid beetles (order Coleoptera) is significant because the virus is vectored by phylogenetically unrelated species, with a distinct developmental biology, ecology, and feeding strategies.

Because field sampling has revealed the occurrence of different thrips species in cornfields in Hawaii (unpublished), the question is raised whether other thrips species may be competent MCMV vectors. From an epidemiological perspective it would be interesting to understand whether thrips may be implicated in the epidemiology of MCMV in corn growing areas other than Hawaii. For instance, in Nebraska and Kansas there have been indication that MCMV spreads very rapidly during the maize-growing season, suggesting that beetles may not the major source of virus spread (Scheets 2008). Although MCMV tends to reappear in previously infected fields, the virus has not been demonstrated to be soil, water or fungus-borne. Recently, outbreak of MCMV has been reported in corn-growing regions of Yunnan, China (Xie et al. 2011). However, to our understanding it is not known what vector species may be implicated.

In summary, our research provides evidence that MCMV is transmitted by corn thrips semipersistently. This appears to be a unique feature among thrips-vectored plant viruses. Although the mechanisms underling this type of interaction are currently not understood, we propose that MCMV can become a valuable model system to dissect and contrast the viral determinants that mediate transmission by arthropod

vectors with piercing sucking (corn thrips) and chewing mouthparts (chrysomelid beetles).

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