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Delivery of maize mosaic virus to planthopper vectors by microinjection increases infection efficiency and facilitates functional genomics experiments in the vector

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ABSTRACT

The corn planthopper, *Peregrinus maidis,* not only causes direct damage to plants by feeding, but also transmits maize mosaic virus (MMV) to the plant hosts. The virus is transmitted in a propagative manner but the acquisition of MMV by the vector feeding on infected plants can result in low acquisition and inoculation efficiency. Here, we increased the acquisition efficiency by delivering the virus directly into the hemocoel through microinjection, which resulted in efficient virus infection of the insect and transmission to maize. We found that delivery of virus by injection of 10 ng MMV (50 nl, 200 μg/ml virions) into *P. maidis* resulted in 93% transmission efficiency. In dose-response experiments, MMV abundance in insects and transmission efficiency decreased as the amount of virus inoculum delivered into the hemocoel was reduced. Examination of virus distribution in the vector using immunolabeling and confocal microscopy revealed similar tissue distributions in the injected insects when compared to those of previous studies using feeding on plants for virus acquisition. The utility of virus inoculation by microinjection for functional analysis in virus-vector interaction was explored. Co-microinjection of MMV virions and the dsRNA of *PI3Kδ* (a transcript that is less abundant in MMV-infected insects), resulted in a reduction in *PI3Kδ* expression and higher virus titers in *P. maidis*. These findings demonstrated that virus microinjection is a robust method for obtaining large numbers of infected planthoppers that are competent in transmitting MMV and, in combination with RNAi, could significantly facilitate the functional analysis of *P. maidis*-MMV interactions.

1. Introduction

The corn planthopper, *Peregrinus maidis* (Ashmead) (Hemiptera: Delphacidae), is a destructive insect pest of maize (*Zea mays*) and sorghum (*Sorghum bicolor*) that is widely distributed across tropical, subtropical and temperate regions (Namba and Higa, 1971). *P. maidis* causes damage by feeding on plant tissues via the piercing-sucking mouthparts. Feeding by the insects on the leaf midrib, whorl and sheath also causes stunting and leaf chlorosis, and reduces plant vigor (Thomas, 1913; Fullaway, 1918). Severe infestations can result in basipetal withering of leaves and even death of plants. The insect also indirectly damages plants in two ways: (1) transmission of maize mosaic virus (MMV) (genus: *Nucleorhabdovirus,* family*: Rhabdoviridae*) causes symptoms on plants, like yellow spots or interrupted stripes, bands between and along the fine veins, and stunting; and (2) the copious amounts of honeydew excreted on plant surfaces provide a substrate for sooty mold growth and impair leaf photosynthetic capacity (Chelliah and Basheer, 1965; Borikar and Deshpande, 1978). The combination of damage from feeding and virus infection can significantly reduce crop yields (Nault, 1989; Redinbaugh et al., 2012).

MMV is a negative sense single-stranded RNA virus (-ssRNA) with bullet-shaped virions (Jackson et al., 2005; Reed et al., 2005). The gene order on its genomic -ssRNA is 3`-N, P, 3, M, G, L-5`, in which N encodes the nucleocapsid protein; P, the phosphoprotein; 3, movement protein in plants; M, the matrix protein; G, the glycoprotein; and L the RNA-D ependent RNA polymerase (Redinbaugh and Hogenhout, 2005). MMV is transmitted by *P. maidis* in a persistent propagative manner, i.e., MMV replicates in and systemically invades the insect vector (Nault, 1997). The virus persists in the vector for the duration of the insect's life and the insect is capable of virus transmission after a latent period of 1–3 weeks (Ammar et al., 2009 and unpublished data). MMV initially infects *P. maidis* in the midgut and then spreads throughout the

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alimentary tract, hemocytes, compound eyes, nerve ganglia, visceral muscle, tracheae, salivary glands and other tissues (Ammar and Hogenhout, 2008). Due to the propagative nature of the virus, infected planthoppers have the potential to transmit MMV for an extended period of time, and one insect could effectively inoculate many individual plants.

For MMV to be successfully transmitted by the corn planthopper, the virus must traverse multiple barriers in the vector. In the initial acquisition steps, the virus must enter and replicate in the midgut epithelial cells, then spread to other gut cells and move beyond the gut basal plasma membrane and basal labyrinth. MMV was detected in the hemocytes and neural tissues and it was suggested that the virus may establish a systemic infection in the insect using the hemolymph and/or neural system routes (Ammar and Hogenhout, 2008). Regardless of the route, movement to, and infection of, the salivary glands is essential for vector transmission to occur (Hogenhout et al., 2003). Along its dissemination path, the virus moves through the gut barriers, evades the insect's innate immune defense, and enters and traverses the salivary gland barriers, before it exits the salivary canal to finally be delivered into the plant cells by its feeding inoculum. Falk and Tsai (1985) reported that MMV transmission efficiency of *P. maidis,* after feeding acquisition, could reach up to 42%, while injection of insects with virus increased infection to 85%, suggesting that bypassing insect tissue barriers could increase virus infection.

Although the molecular response of *P. maidis* to MMV is largely uncharacterized, it has been hypothesized that, like other rhabdoviruses, MMV has an ability to escape vector cell immune defenses to gain virus entry, replication and movement. For *Vesicular stomatitis virus* (VSV), an animal-infecting rhabdovirus, the defense pathways of the animal hosts are negatively modulated, and the PI3K/Akt-mediated programmed cell death (apoptosis) pathway was inhibited (Desforges et al., 2002). In general, PI3K/Akt was described as a virus-induced signaling module (Ehrhardt, 2011), and infections by a variety of retroviruses, DNA viruses and RNA viruses activate PI3K and Akt. The induction of Akt signaling can lead to the activation of the host's innate immune genes, such as the inflammatory cytokine genes (Dunn and Connor, 2012). However, some viruses appear to inhibit the PI3K/Akt pathway (Dunn and Connor, 2011; Shelly et al., 2009). Previously, it was proposed that MMV might also avoid or inhibit PI3K/Akt signaling in the midgut of *P. maidis* during infection (Whitfield et al., 2011). Therefore, an RNAi-mediated knockdown of the PI3K transcripts might facilitate MMV infection.

The goal of this study was to develop a robust and reliable methodology for delivering MMV into *P. maidis* that would enable functional genomics experimentation for this pathosystem. The effects of MMV microinjection and dsRNA delivery on the enhancement of MMV infection and transmission efficiencies were tested in this study. To ensure that microinjection delivery of MMV is a valid alternative to feeding acquisition from plants, we also compared the dissemination route of the artificial virus delivery system to the published descriptions of virus movement in naturally infected *P. maidis*.

2. Materials and methods

2.1. Insect rearing

P. maidis was reared on 3-week old maize plants (*cv.* Early Sunglow, Syngenta Inc., Greensboro, NC) at the four to six leaves stage, and kept in insect-proof cages covered by 46-micron nylon-mesh fabric (customized insect-proof 12″ × 12″ × 24″cage, Bioquip Inc, Compton, CA) in a plant growth chamber at a 24 ± 1 °C and 14L:10D photoperiod. In order to synchronize the insect vector's developmental stages, mature adults were placed on fresh maize plants for a 24-hr oviposition period, after which the adults were removed from the plants, and the deposited nymphs were moved to a new cage with fresh maize plants. The insects were also similarly transferred after each molt, and the developmental stages were confirmed by microscopy.

2.2. MMV purification

MMV-infected maize leaf tissue (≈ 64 g) was used for virus purification (Jackson and Wagner, 1998 & Ammar et al., 2005). The purified viral particles were resuspended in 500 µl phosphate buffered saline (PBS) containing 10% glycerol and stored in a liquid nitrogen tank. The protein concentration of the purified MMV preparations was measured by the BCA protein assay (Thermo Fisher Scientific Inc. Carlsbad, CA, USA).

2.3. Serological detection of MMV replication in P. maidis after injection

Purified MMV (virus protein conc. 250 µg/ml) virions were diluted into three concentrations (250 µg/ml (high), 25 µg/ml (medium), and 2.5 µg/ml (low). Eight newly emerged adult planthoppers (less than 24 h-old) were collected and anesthetized by chilling on ice. The immobilized insects were then placed in an injection arena (10-cm-diameter petri dish containing a layer of 1% agarose). The connective membrane between the third and fourth dorsal abdominal segments of each insect was pierced once to deliver 50 nl of each treatment at 10 nL/sec speed using a Nanoinjector II (Drummond Scientific, Broomall, PA). MMV accumulation after microinjection was monitored every other day. After injection, insects from each treatment (concentration of MMV) were reared on a pot of one-week-old maize seedlings (cv. Early Sunglow) at 24 ± 1 °C and a 14L:10D photoperiod, and the seedling pots were covered with a 7.62 cm (diameter) × 30.48 cm (length) clear plastic tube (Uline Inc. WI, USA) capped with insect-proof mesh. Three samples of each MMV concentration treatment were collected at 0-, 3-, 6-, 9- and 12-day post-injection for RT-qPCR to quantify MMV relative abundance. Total RNA was extracted from each sample (three insects per sample) by homogenizing in 200 µl of TRIzol™ reagent (Thermo Fisher Scientific Inc. Carlsbad, CA, USA), according to the manufacturer's RNA extraction guide (Chomczynski, 1993).

Three samples (three insects per sample) from each concentration of MMV injected insects were also collected at each time point (0-, 3-, 6-, 12- and 15-day post-injection) to analyze the amount of viral protein using western blotting. The samples were homogenized with 250 µl lysis buffer (50 mM Tris − HCl, 150 mM NaCl, 1 mM EDTA, 1% NP40) containing protease inhibitor cocktail (Roche Inc. Indianapolis, IN), centrifuged (5000 rpm) at 4℃ for 5 min and the supernatant (5.0 µl of each sample) was loaded onto SDS-PAGE gels. The amount of viral protein was detected using goat anti-MMV-P (phosphoprotein) serum (from Margaret Redinbaugh, USDA-ARS, Wooster, OH). β-actin served as a loading control and was detected with monoclonal anti-β-actin-HRP conjugated antibody (Cat No: A3854, Sigma-Aldrich, St. Louis, MO). All experiments were repeated twice.

2.4. Quantitative real time RT-PCR of MMV-N

MMV N (nucleocapsid gene) transcript levels were monitored from three samples of each treatment by RT-qPCR after MMV microinjection. Total RNA quantity was analyzed at 260 nm with a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific Inc. Carlsbad, CA, USA). The first strand cDNA (10 µl) of each sample was synthesized from 0.5 µg total RNA using the ThermoScientific Verso cDNA synthesis kit with RT-enhancer (Thermo Fisher Scientific Inc. Carlsbad, CA, USA) to remove residual DNA contamination. Around 5 ng of cDNA and the *MMV-N* gene-specific primer pair (Barandoc-Alviar et al., 2016) were used in a 10 µl SYBR Green Supermix reaction (Bio-Rad Inc. Hercules, CA). Quantitative PCR was performed with a two-step amplification protocol with 40 cycles at 95 °C for 30 s, 56 °C for 30 s using a Bio-Rad IQ thermocycler (Bio-Rad Laboratories Inc. Hercules, CA, USA). To make comparisons among different time points and different MMV concentration treatments, the abundance of *MMV-N* RNA was

normalized to *RPL10* (Yao et al., 2013), using the equation $2^{(Ct\ (MMV\text{-}N)\text{-}Ct\ (RPL10))}$. Herein, we refer to the abundance of target transcripts as normalized abundance.

2.5. Mortality of P. maidis after MMV injection

After MMV microinjection, time course experiments (a 3-week period) were conducted to determine the effects of different concentrations (200 μg/ml, 20 μg/ml, 2.0 μg/ml) of MMV injection on nymph mortality. In total, sixty 5th instar nymphs were injected with 50 nl of MMV (158 μg/ml, 20 μg/ml, 2.0 μg/ml, 0 μg/ml) or H2O, respectively. Dead insects were recorded every other day, and the mortality tested by comparing the number of nymphs that died after MMV injection to those that died from the virus mock or PBS microinjections (control). Each treatment consisted of three replicates of 20 nymphs. The experiment was repeated twice.

2.6. MMV transmission assay

Three concentrations (200 μg/ml, 20 μg/ml and 2.0 μg/ml) of purified MMV injected *P. maidis* adults were also used to test virus transmission efficiency. In this experiment, 50 nl of purified MMV virions (200 μg/ml, 20 μg/ml and 2.0 μg/ml) were injected into newly emerged adults (≤ 24-hr old). After microinjection, 20 adults of each concentration (or treatment) were reared on young maize plants for 3-days to eliminate death resulting from microinjection damage. Then the living insects were individually transferred onto individual maize seedling (one insect per plant), and each seedling was covered with plastic tube that was capped with insect-proof mesh. After a 10-day inoculation access period (IAP), the living insects from each plant were removed and the maize seedlings allowed to grow until MMV symptoms were apparent (≤7-days). The experiment was performed three times. During the 10-day IAP, the survival of insects on each individual seedling was recorded daily, and seedlings with insects that survived for more than 7-days were saved. If the insect died before day 7, this seedling was removed from the transmission assay. Virus infection of the inoculated plants was determined by DAS-ELISA using commercial antibodies against MMV (Agdia, Inc. Elkhart, IN, USA). Maize leaf tissues (≈ 0.1 g) were ground in 1 ml cold general extraction buffer (4 °C) and ELISA was conducted as indicated by the manufacturer's protocol. Absorbance was read at 405 nm, and the samples with absorbance values higher than 3 times the mean absorbance values of controls (virus-free leaf tissues) were considered as positive (Sutula et al., 1986).

2.7. Immunohistochemistry

In total, 60 insects of each treatment (PBS and MMV microinjections) were used to detect MMV distribution by immunostaining of cryosections, tissues and hemocyte. Newly emerged *P. maidis* adults (≤24 h old) were injected with 50 nl MMV virions (200 μg/ml) or PBS (as a negative control). After microinjection, insects were incubated on a young maize plant at 24 ± 1℃ and the live insects were collected at 3-, 6-, 9- and 12-day post-microinjection for examination of MMV distribution.

2.7.1. Cryosection sample preparation

After PBS and MMV microinjection, the whole bodies of 10 insects per treatment were fixed in 4% paraformaldehyde in PBS at 4℃ overnight. Prior to fixation, the legs and wings of each insect were removed, and a small tear was introduced in the thoracic cuticle and abdomen to allow penetration of the fixation solution. The fixed insects were infiltrated with the increasing sucrose concentrations (12, 15, 18 and 20% sucrose in PBS) at 4℃. The infiltrated samples were placed in a drop of Tissue-Tek® O.C.T™ compound (Sakura Finetek USA Inc. Torrance, CA, USA) on a small piece of filter paper and then frozen by dropping them into a tube of 2-methylbutane that was pre-cooled with liquid nitrogen. Longitudinal sections (12 μm) were made with a Leica 3050 cryosection station at -20℃. The sections were attached on a superfrost plus slide (Thermo Fisher Scientific Inc. Carlsbad, CA, USA) and rinsed three times with PBST (PBS with 0.1% Tween 20). The sections were then permeabilized by incubating in 0.1% Triton X-100 in Tris buffered saline for 10–15 min. Sections were blocked for 2-hrs in PBST containing 2% bovine serum albumin. After blocking, sections were incubated for 2-hrs with rabbit-anti MMV antiserum (1:400 dilution) and rinsed 3 times ×5 min with PBST before incubation for 1-hr with goat anti-rabbit IgG labeled with Alexa Fluor 488 (1:600 dilution) (Thermo Fisher Scientific Inc. Carlsbad, CA, USA).

2.7.2. Hemocyte smear preparation

Sixteen PBS and MMV microinjected insects were used for hemolymph smear preparations, respectively. Each insect body was squeezed with sharp forceps and the hemolymph droplets from openings of the removed legs were carefully collected with fine pipette tips. The droplets from each insect were mixed with 10 μl PBS buffer and loaded onto one well of 10-well polytetrafluoroethylene coated slide (Electron Microscopy Sciences Inc. Hatfield, PA). In total, the hemolymph from 8 insects was collected and loaded on one slide and two slides were prepared for PBS or MMV microinjection, respectively. The hemocytes were fixed with 4% paraformaldehyde at 4 ˚C overnight, rinsed and permeabilized for 30 min in PBT (PBS plus 0.1% Triton X-100) before blocking for 1 h and incubation with rabbit anti-MMV antiserum (1:400 dilution) for 2 h. After 5 min rinses for 5 times with PBST, the samples were incubated for 1 h with goat anti-rabbit IgG labeled Alexa Fluor 488 (1:400 dilution in PBS), and then washed with PBST (5 times ×5 min).

2.7.3. Whole-mount tissue

Thirty MMV and PBS microinjected planthoppers were used for tissue dissection. The dissected tissues (salivary gland, gut, and reproduction organs) were fixed with 4% paraformaldehyde for 2-hrs following the Ammar procedure with minor modifications (Ammar and Hogenhout, 2008), and blocked with PBST containing 2% bovine serum albumin. After blocking, the tissues were incubated at 4℃ with rabbit-anti MMV antiserum (1:400 dilution in BSA blocking buffer) overnight. After incubation, the tissues were rinsed 5 times for 5 min each with PBST and incubated for 1 h with goat anti-rabbit IgG labeled with Alexa Fluor 488 (1:600 dilution in BSA blocking buffer). Finally, the tissues were washed once with PBS and incubated with Phalloidin-Alexa 594 conjugate (4units/ml) in BSA blocking solution for 1 h. After incubation, the tissues were washed (5 times ×5 min) with PBST.

2.7.4. Confocal imaging

Before sealing, all cryosections, hemocyte and dissected tissue samples were incubated for 15 min with 2 μg/ml DAPI (4′, 6-diamidino-2-phenylindole) to stain the cell nuclei blue. After DAPI incubation, all samples were rinsed with PBST (3 times ×5 min), covered with mounting buffer (50% glycerol in PBS 1X buffer), and sealed with nail polish. Finally, all samples were observed with a Zeiss LSM 510 META laser scanning confocal microscope using excitation wavelengths of 405 nm (DAPI) and 488 nm (MMV) with a 10× Plan-Neofluar objective with a 0.3 numerical aperture (NA) or a 40× Plan-Neofluar oil objective with a 1.3 NA.

2.8. cDNA sequencing of PI3K δ subunit gene

A partial cDNA sequence (528 bp) of *PI3Kδ* subunit was identified from the *P. maidis* gut- specific EST library using Blast2GO (Whitfield et al., 2011). To obtain a full-length open reading frame (ORF) sequence for the *PI3Kδ* subunit gene, 5` rapid amplifications of cDNA ends (5` RACE) (Clontech SMART™ RACE cDNA amplification kit, Mountain View, CA) were performed on 1 μg total RNA extracted from a group of five 5th instar nymphs using the RNeasy mini kit (Qiagen

Inc. Valencia, CA, USA). Touchdown PCR was performed with one *PI3K-δ* subunit gene-specific primer (5`- ACAGCTGTCCAGTGCGTTTG ACCAT-3`) and a universal primer (5`-CTAATACGACTCACTATAGGG CAAGCAGTGGTATCAACGCAGAGT–3′) provided with the Clontech RACE cDNA kit. The resulting PCR products were sub-cloned using the Invitrogen TA-vector system (Thermo Fisher Scientific Inc. Carlsbad, CA, USA) and sequenced using the ABI 3700 DNA sequencer at the Kansas State University DNA Sequencing Facility (Manhattan, KS, USA).

Multiple PI3Kδ catalytic subunit amino acid sequence alignments of *PI3Kδ* of *P. maidis*) were performed with ClustalW2 (www.ebi.ac.uk) sequences from *Homo sapiens (PIK3CD*, GenBank accession No: NM_005026), *Mus musculus* (*Pik3cd*, GenBank accession No: NM_001164052), *Drosophila melanogaster* (*Pi3K92E*, GenBank accession No: NM_142645), *Aedes aegypti* (GenBank accession No: XP_001662660), *Acyrthosiphon pisum* (GenBank accession No: XP_001946655) and *Apis mellifera* (GenBank accession No: XP_623897).

2.9. PI3Kδ gene expression levels after MMV infection

PI3Kδ expression levels were monitored individually in MMV positive adults after feeding acquisition. In this experiment, MMV free adults were placed on MMV-infected maize plants and allowed to lay eggs. After hatching, the nymphs were reared on MMV-infected maize plants until emerging as adults (the average MMV acquisition period ≥15 days). *MMV-N* transcripts were individually detected using *MMV-N* qPCR primers (Table 1). If the *MMV-N* transcript level of one insect was detectable by RT-PCR, this insect was defined as MMV positive and the cDNA of this insect was saved for *PI3Kδ* subunit transcription detection using qPCR primers (Table 1). In total, we screened more than 80 insects collected from infected maize plants and identified 20 MMV positive insects. Twenty MMV-free insects from MMV-free maize plants were used as controls. To make *PI3Kδ* expression comparisons between MMV-infected and non-infected insects, the abundance of *PI3Kδ* was normalized to *RPL10* using the equation $2^{\wedge\ (Ct\ (PI3K\delta)\text{-}Ct\ (RPL10))}$.

2.10. dsRNA synthesis

Total RNA (1.0 μg) of *P. maidis* was used for cDNA synthesis by Thermo Scientific Verso cDNA Synthesis kit (Waltham, MA, USA). The target sequence fragments were amplified from cDNA template by PCR using template-specific primers (Table 1) conjugated with the 23 bp T7 RNA polymerase promoter. GFP fragments were amplified from the pHsp70-GFP-polyA vector. For dsRNA synthesis, purification, quality and quantity tests followed the procedure of Yao et al (2013).

2.11. dsRNA and MMV delivery by injection

A time-course experiment was conducted to determine the efficiency of dsRNA injection on target gene silencing. Newly emerged *P. maidis* adults (sixty insects) were injected with 50 nl *dsRNA-PI3Kδ* (2 mg/ml), *dsRNA-GFP* (2 mg/ml) and H_2O as the blank control. The experiment was performed twice with three experimental replicates per treatment (20 insects/per treatment). The injected adults were reared on healthy maize plants at 24 ± 1 °C, 14/10 h light/dark in insect rearing cages. At 1-, 3-, 6-, 12-days post-injection, three live insects were collected from each replicate and homogenized in 200 μl Trizol reagent (3 independent replicates per treatment), and processed for *PI3Kδ* subunit transcript level analysis by RT-qPCR. The relative expression ratio of *PI3Kδ* was normalized to *RPL10* and analyzed by using $2^{-\triangle\triangle Ct}$ (equation: $2^{\ dsRN\ A\ (Ct\ (PI3K\delta)\text{-}Ct\ (RPL10))}\ /\ 2^{H2O\ (Ct\ (PI3K\delta)\text{-}Ct\ (RPL10))}$).

To investigate whether *PI3Kδ* silencing influences MMV replication efficiency in *P. maidis* after injection, the new emerged adults were injected with a mixture of purified virus and dsRNA. In the experiment, 180 newly emerged adults were injected with 50 nl mixtures of MMV-*dsPI3K*, MMV-*dsGFP* or MMV-H_2O (20 insects/per replicate, and 60 insects/per treatment). The virus was concentration injected was 100 μg/ml of purified MMV and dsRNA concentrations of 2 mg/ml were used in the experiments. The injected adults were placed on the caged maize plants and reared at 24 ± 1 °C and 14/10 h light/dark. At 3-, 6- and 9-days post-injection, total RNAs of three pooled insects from each replicate were extracted using Trizol reagent (n = 3 independent replicates per treatment) and processed for *MMV-N* transcript level analysis by RT-qPCR. To make comparisons across treatments, the abundance of *MMV-N* detected by RT-qPCR was normalized to *RPL10* using the equation $2^{(Ct\ (MMV\text{-}N)\text{-}Ct\ (RPL10))}$.

2.12. Statistical analyses

The normalized *PI3Kδ, MMV-N* transcript levels were log-transformed, and the percentage of mortalities were arcsine square root-transformed (Yao et al., 2013). After transformation, all data were first tested for normal distribution with Minitab software. SAS procedure PROC GLMMIX, PROC MIXED and the LSMEANS statement were used to evaluate: i) MMV transmission efficiency after different concentrations of injected viral particles; ii) differences in mortality of *P. maidis* after MMV injection; iii) *PI3Kδ* transcript levels in *P. maidis* after MMV injection; and iv) the significant effects of the *dsRNA-PI3Kδ* treatment on the abundance of *PI3Kδ* transcripts. Pearsons correlation of initial injected concentrations with the normalized abundance of the *MMV-N* transcript were analyzed by SAS procedures (PROC CORR) and (PROC REG).

3. Results

3.1. MMV relative abundance increased and persisted after microinjection

A dose-response experiment was conducted to examine MMV replication in *P. maidis* after microinjection with 50 nl of MMV (250 μg/ml, 25 μg/ml, and 2.5 μg/ml). MMV injected adults were collected every other day until 15-day after injection. RT-qPCR analysis revealed

Table 1
Primers used for dsRNA synthesis and quantitative reverse transcription PCR (RT-qPCR).

Gene	Primer usage	Sequence (5' – 3')	Product length	Reference
PI3K-δ	dsRNA synthesis	T7F: TAATACGACTCACTATAGGGATCCAGAGGAGCCCAAACTT F: ATCCAGAGGAGCCCAAACTT T7R: TAATACGACTCACTATAGGGTCGTCCAAAGTGACGAAAGA R: TCGTCCAAAGTGACGAAAGA	347bp	
PI3K-δ	RT-qPCR	F: AAATTCCCATCTTCTCTG R: AATAGCAAAGTTGTCCTC	128bp	
MMV-N	RT-qPCR	F: GAGCATCTGGTAGAGGAG R: CATAGGTTCAGGAGCGTAT	126bp	Barandoc-Alviar et al., 2016
RPL10	RT-qPCR	F: CGAAGAAATGGGGTTTCA R: ACTCGCCTTGTATCTGTCG	105bp	Yao et al., 2013

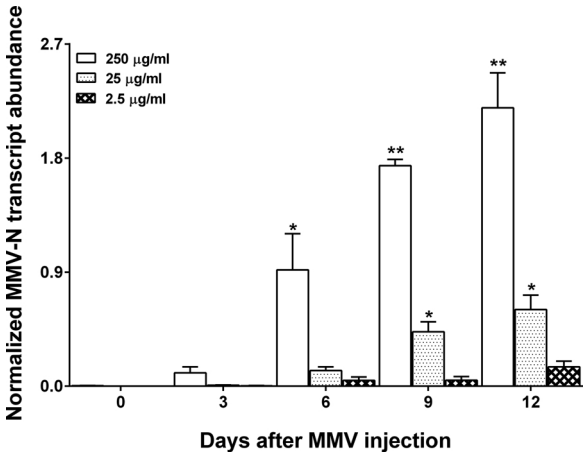

Fig. 1. Time course analysis of accumulation of MMV nucleocapsid RNA in *P. maidis* after microinjection of purified virus. Newly emerged adults (≤ 24-hrs since nymphal eclosion) were injected with three different concentrations of MMV and insects were collected at 0, 3, 6, 9, and 12 days post-injection. Transcripts were quantified using qRT-PCR. The abundance of *MMV-N* transcripts was normalized to *P. maidis RPL10* transcripts. Each bar represents the mean and standard error (SE) of three experimental replicates, and each replicate consists of a group of three insects. The asterisks indicate that the treatment means differ significantly (**p ≤ 0.01, *p ≤ 0.05).

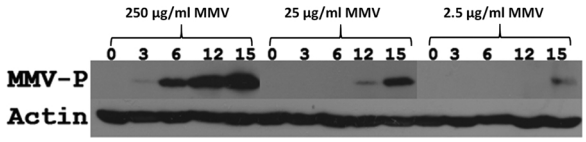

Fig. 2. Detection of MMV phosphoprotein (P) by Western blot analysis of *P. maidis* at 0-, 3-, 6-, 12- and 15-days after MMV injection. Newly emerged adults (≤ 24-hrs) were injected with 50 nl of three different concentrations (250 μg/ml, 25 μg/ml, and 2.5 μg/ml) of MMV virions. MMV phosphoprotein in groups of three insects was detected using an anti-MMV-P goat serum, and β-actin expression was used as an internal reference by labeling the protein with the anti-β-actin-HRP conjugated antibody.

that all microinjected insects were MMV positive (100%), and that *MMV-N* abundance gradually increased over incubation time. The 250 μg/ml MMV injection resulted in the highest abundance of *MMV-N* transcripts compared to the 25 μg/ml and 2.5 μg/ml injections over the 12-day sampling period (Fig. 1). At 12-days post-injection, the abundance of *MMV-N* transcripts in insects with 250 μg/ml injection were around 4-fold higher than those of the 25 μg/ml injections and 14-fold higher than the 2.5 μg/ml injections. Western blotting also showed that the quantities of MMV P protein increased over time, with the 250 μg/ml concentrations resulting in higher protein content than those of the 25 μg/ml and 2.5 μg /ml injections. The lowest (2.5 μg/ml) MMV injected insects only exhibited a very faint band at 15-days post-injection (Fig. 2).

Pearson correlation analyses revealed that the concentrations of the initial injected viral particles along with the time course were positively correlated with the abundance of MMV-N transcripts in *P. maidis*. The correlation coefficients of each initial MMV concentration (250 μg/ml, 25 μg/ml and 2.5 μg/ml) are 0.9145 (r^2 = 0.8327, *p-value* < 0.001), 0.9125 (r^2 = 0.8267, *p-value* < 0.001), and 0.7649 (r^2 = 0.58502, *p-value* = 0.001), respectively. These results indicate that MMV replicates quickly after microinjection with a negligible latency period in *P. maidis*, because the abundance of the *MMV-N* transcripts and MMV-P proteins increased significantly as the incubation periods (as short as 6 days) were extended. In summary as the incubation times increased, higher virus abundances were detected by both RT-qPCR (Fig. 1) and western blotting (Fig. 2).

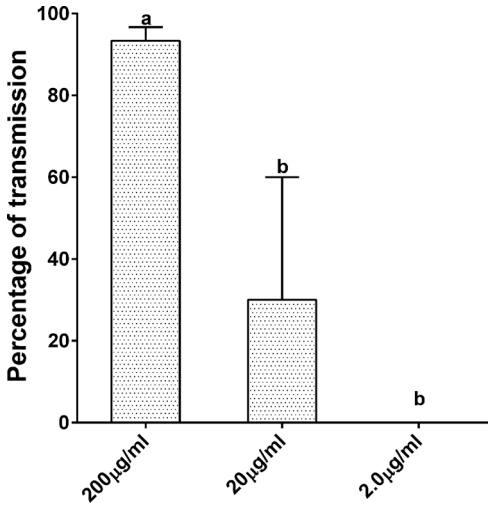

Fig. 3. The percentage of ELISA-positive maize plants obtained after inoculation access periods with MMV-injected *P. maidis*. Fifty nanoliters of three different concentrations (200 μg/ml, 20 μg/ml and 2.0 μg/ml) of MMV were injected into *P. maidis* adults. Three days after injection, these MMV-injected insects were used to transmit MMV to healthy maize plants. Each bar represents the mean and standard error (SE) of three experimental replicates, and each replicate consists of a group of 10 plants. Different letters represent significant differences in transmission efficiency between treatments (p ≤ 0.05).

3.2. MMV transmission assay

P. maidis young adults microinjected with MMV (200 μg/ml, 20 μg/ml, and 2.0 μg/ml, respectively) were used for inoculation of MMV back to maize seedlings. Significant differences (*p-value* = 0.032) were observed in transmission efficiencies amongst planthoppers (20 adults), injected with 50 nl of different MMV concentrations. Insects injected with 200 μg/ml had 93% transmission efficiency, and those injected with 20 μg/ml had approximately 30% transmission, whereas insects injected with 2.0 μg/ml MMV failed to transmit detectable virus to the plants after 10-day feeding periods (Fig. 3).

3.3. Mortality of nymphs injected with MMV

To determine the lethality of MMV to *P. maidis* after injection, time-course experiments were conducted to determine the effects of MMV injection on nymph survival. The survival of nymphs injected with 50 nl of two controls (PBS and virus mock) and MMV (200 μg/ml, 20 μg/ml, and 2.0 μg/ml) were determined at 3-day intervals. No significant differences (*p-value* = 0.17) in survivorship of *P. maidis* nymphs were found amongst five different treatments in the 21-day experiment (Fig. 4), which indicates that MMV infection may not have lethal effects on *P. maidis*.

3.4. MMV distribution in P. maidis after microinjection

To understand the kinetics of MMV movement and distribution in *P. maidis* after microinjection, immunostaining of cryosections, tissues and hemocytes were performed by confocal microscopy. After MMV injection, hemocytes were the first target of MMV infection. No differences in the percentage of infected hemocytes were found among four different testing time points (3, 6, and 12-day post-injection). On average, one third of the hemocytes were infected with MMV after injection (Table 2), although the intensity of MMV signals in individual infected hemocytes increased as the incubation time increased from 3- to 12-days post-injection (Fig. 5).

After microinjection, MMV virions could be detected in the foreguts, midguts and tracheas of insects as early as 3-days post-infection

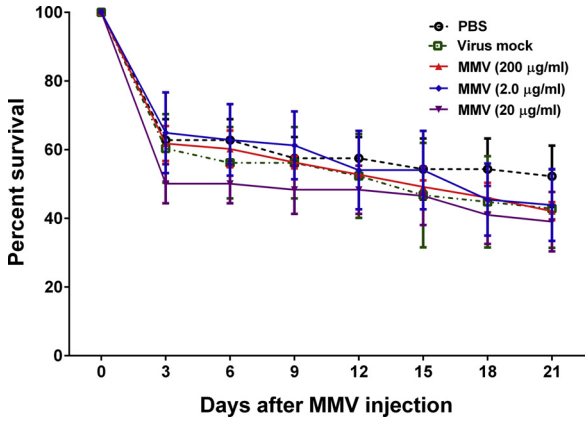

Fig. 4. *P. maidis* nymphal mortality after MMV injection. Fifth instar nymphs were injected with 50 nl of different concentrations of MMV particles, mock virus purification or PBS. Each point and the associated bar represent the mean ± standard error of three experimental replicates, with 20 insects per replicate. Insect mortality was monitored every other day up to 21 days post-injection.

Table 2
Detection of MMV in various tissues by immunofluorescence microscopy at 3-, 6-, 9-, and 12-days after injection of purified MMV (10 ng).

Organs/tissues	Percentage of infected tissue (number infected/number examined)*			
	3-dpi	6-dpi	9-dpi	12-dpi
Hemocytes[&]	29.5 ± 4%	38.4 ± 5%	No data	33.4 ± 4.8%
Compound eyes	0% (0/13)	0% (0/13)	No data	100% (13/13)
Salivary glands	0% (0/15)	6.7% (1/15)	80% (12/15)	100% (15/15)
Foregut	11% (1/9)	88.9% (8/9)	100% (9/9)	100% (9/9)
Midgut	9.1% (1/11)	90.9% (10/11)	100% (11/11)	100% (11/11)
Hindgut	9.1% (1/11)	63.6% (7/11)	100% (11/11)	100% (11/11)
Reproductive organs [#]	0% (0/13)	69.2% (9/13)	100% (13/13)	100% (12/12)

"*" All data are from two experiments. "dpi" indicates the days post-injection with MMV.
"&"Hemocyte infection is presented as a percentage because hemolymph from individual insects was imaged on individual slides and the number of hemocytes collected from each insect varied.
"#" MMV particles were detected on female membranes of ovary and genital chamber.

(Table 2). MMV virions moved into salivary glands and female ovary membranes by 6-days post-infection, and to compound eyes by 9-days post-infection. The intensity of MMV immunolabeling in these organs increased as the incubation times increased from 6- to 12-days post-infection as described for the hemocytes (Fig. 6). Finally, MMV virions were detected in all tested tissues (Fig. 6 and Table 2).

3.5. cDNA sequence of PI3Kδ

The full-length cDNA sequence of the *PI3Kδ* catalytic subunit was obtained from 5` RACE and submitted to NCBI GenBank (GenBank accession: KY707127). The predicted ORF for *PI3Kδ* is 3491 nt, which encodes a 1083 amino acid protein belonging to the PI3Kc superfamily. The predicted amino acid sequence of *P. maidis* PI3Kδ shared only 45–67% identities with PI3Kδ from *M. musculus* (45%), *H. sapiens* (46%), *D. melanogaster* (48%), *A. aegypti* (55%), *A. pisum* (66%) and *A. mellifera* (67%). However, *P. maidis PI3Kδ* shares a conserved catalytic loop (GVADRHSDN) with three conserved residues (^{930}Asp, ^{931}Arg and ^{935}Asn), a conserved activation loop (A-loop, DFGHILGHFKEKFGFRRERVPFVLT) with two conserved positively charged residues (^{957}Lys and ^{966}Arg) (Pirola et al., 2001) and the well-conserved 3`-terminus characteristic of *PI3Kδ* (Supplementary Fig. 1).

3.6. PI3Kδ transcript level in MMV-infected P. maidis transcript level in MMV-infected P. maidis

To determine whether MMV infection affects *PI3Kδ* gene expression in *P. maidis*, the transcript levels were monitored individually in MMV positive insects. In this study, we found that transcript levels of *PI3Kδ* were significantly lower in MMV-infected insects when compared to uninfected insects (*p-value* = 0.004) (Fig. 7).

3.7. PI3Kδ knockdown after dsRNA injection

To determine whether *dsRNA-PI3Kδ* injection could knockdown *PI3Kδ* transcript levels, we injected *dsRNA-PI3Kδ*, *dsRNA-GFP* and H_2O into *P. maidis* adults. On average, injection of adults with *dsRNA-PI3Kδ* resulted in significant reduction of *PI3Kδ* transcript ranging from 12- to 36-fold during the 12-day sampling period. There were no significant differences in *PI3Kδ* transcript levels at different time-points after *dsRNA-GFP* injection, and the levels were significantly reduced at all times (1-, 3-, 6-, and 12-days) post-injection (Fig. 8).

3.8. MMV transcriptional level changes after dsRNA-PI3Kδ knockdown

To determine whether knocking down *PI3Kδ* expression alters MMV accumulation in insects, we co-injected the planthoppers with different concentrations of PI3K dsRNA and MMV. The treatments included the following combinations of dsRNA and purified virus: MMV + *dsRNA-PI3Kδ* (MMV-*dsPI3Kδ*), MMV + H_2O (MMV-H_2O) and MMV + *dsRNA-GFP* (MMV-*dsGFP*), and the two negative controls: MMV-*dsGFP* and MMV-H_2O injection. The abundance of *MMV-N* was analyzed by RT-qPCR over 9 days. The abundance of *MMV-N* in the three treatment groups increased over 3-, 6- and 9-days post-injection, Fig. 9). No significant differences in *MMV-N* abundance were found between MMV-H_2O and MMV-*dsGFP* injected insects at any of the time points. At 6-days post-injection, significant differences in *MMV-N* abundance were not detected between the two controls and MMV-*PI3Kδ* injection (*p-value* = 0.40 and 0.42, respectively). However, a significant difference in *MMV-N* abundance was found at 3- days post-injection with MMV-*dsGFP* and MMV-*dsPI3Kδ* (*p-value* = 0.017), 9-days (*p value* = 0.007) or between MMV-H_2O and MMV-*dsPI3Kδ* at 3-days (*p-value* = 0.006) or 9-days (0.004). These findings support the hypothesis that PI3K modulates MMV infection and that reductions in *PI3Kδ* transcripts results in higher MMV accumulation.

4. Discussion

4.1. MMV delivery through microinjection

A high degree of vector specificity has been observed for circulative transmission of phytoviruses. For example, barley yellow dwarf virus (BYDV), tomato yellow leaf curl virus (TYLCV), tomato spotted wilt virus (TSWV), rice dwarf virus (RDV) and maize mosaic virus (MMV) are specifically transmitted by aphids, whiteflies, thrips, leafhoppers and planthoppers, respectively (Hogenhout et al., 2008; Whitfield et al., 2015). Leafhopper injections carried out in the 1960's with extracts from potato yellow dwarf virus (PYDV) infected leafhoppers revealed that injected nymphs were able to transmit the virus to a high proportion of plants after an incubation period and suggested that the virus multiplies in the vector (Sinha, 1965). Moreover, injections from tissue and organs of infected leafhoppers showed that PYDV was widely distributed throughout the vector and, provided evidence that the virus multiplied in the vector. Studies conducted with MMV provided more

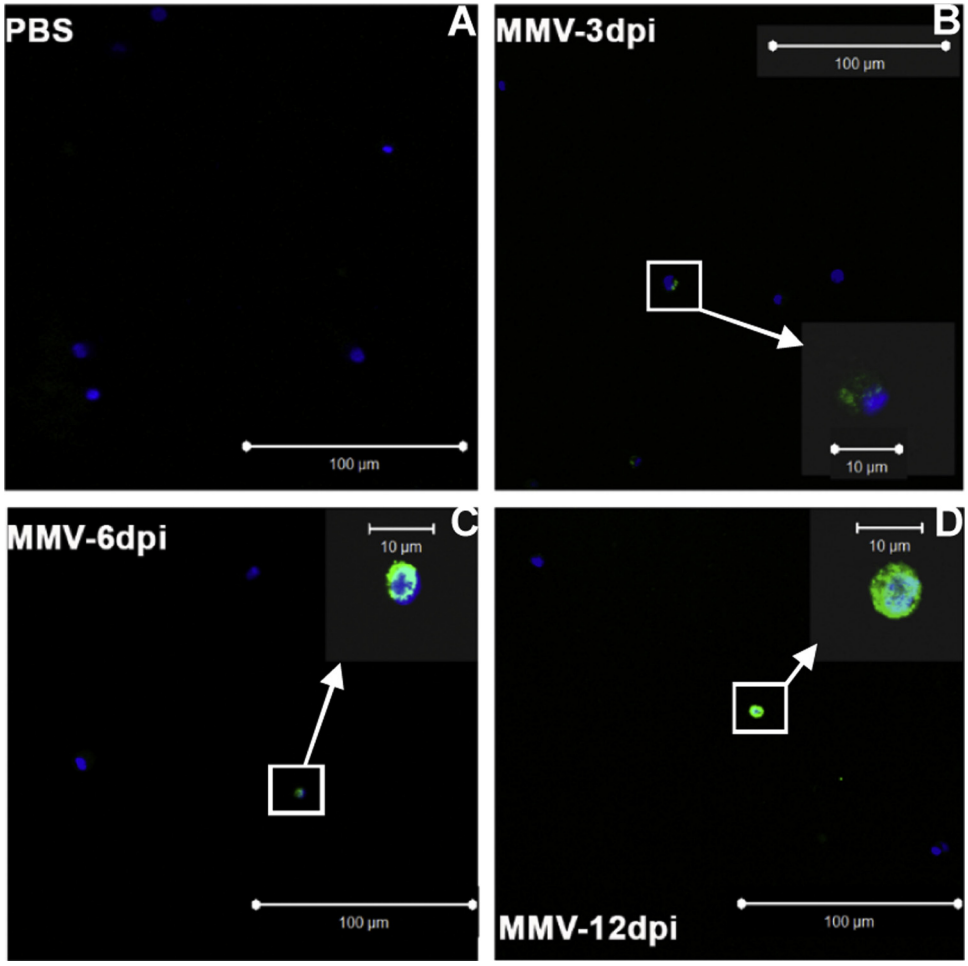

Fig. 5. Progression of MMV infection in *P. maidis* hemocytes at 3, 6- and 12-days post-injection. MMV was detected by immunolabeling and confocal microscopy. Planthopper hemocytes were immunolabeled for MMV with Alexa Fluor (green), and the nuclei were stained with DAPI (blue). (A): Hemocytes from PBS-injected planthoppers at 12-days post-injection are shown as the control (PBS). (B, C, D): MMV-infected hemocytes at 3-, 6-, 12-day post-microinjection are shown in the indicated panels, respectively. Scale bars represent 100 µm in the main panels, and the highlighted hemocytes in each insert panel have 10 µm scale bars (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

extensive descriptions of the infection pathways in the planthopper (reviewed in Ammar et al., 2009; Ammar and Hogenhout, 2008). These studies have shown that during infection with MMV, virus circulates in the planthopper and infects multiple tissues via defined pathways prior to transmission to plants. For successful delivery to a plant host, MMV must pass through entry and exit barriers in the midgut and salivary glands. After *P. maidis* nymphs acquire virus from plants, MMV titer increases over developmental time and peaks at the adult stage (Barandoc-Alviar et al., 2016). Here, we were able to increase MMV infection efficiency by bypassing the gut barrier and delivering purified virus directly into *P. maidis* hemocoel, and we found that the virus dissemination and infection pathway followed previously described routes observed after acquisition via natural infection i.e., feeding on infected plants (Ammar and Hogenhout, 2008). Our results also demonstrate that vector microinjections provide an excellent alternative for functional genomics, because co-delivery of MMV with dsRNAs to target virus-responsive gene silencing illustrate that microinjection is very useful for examining host gene functions during MMV infection. Because of its quantitative delivery possibilities, injection methodology greatly reduces variables inherent in plant feeding by vectors and enables efficient analysis of a wide range of host genes affecting vector infection and virus transmission.

MMV transmission efficiency after feeding acquisition from plants was initially reported to reach up to 42% (Falk and Tsai, 1985). Later, Barandoc-Alviar et al (2016) reported that MMV acquisition by nymphs and adults by feeding reached 63% and 40%, respectively. After MMV acquisition, the insects undergo a latent period before becoming viruliferous for the rest of the life span. In early MMV injection experiments, Falk and Tsai (1985) were able to increase *P. maidis* acquisition and transmission efficiencies up to 76% and 85%, respectively; however, the mortality of the injected insects reached up to 50%. In our study, the modified MMV microinjection method resulted in less than 10% mortality and up to 100% acquisition efficiency in injected insects (Fig. 1). Our results also show that the initial concentration of microinjected MMV is positively correlated with the abundance of viral transcripts and protein and transmission efficiency. Falk and Tsai (1985) also found that the average minimum time between injection and MMV detection in planthoppers was 4 days when measuring virus abundance by ELISA. Our experiments demonstrated that microinjected MMV could be detected as early as three days post-infection by RT-qPCR detection and that the latent period was ≈ 6 days (Table 2). This period is much shorter than MMV acquired by *P. maidis* after feeding on MMV positive plants where 4 to 5 weeks of latency was observed (Ammar and Hogenhout, 2008). Collectively, these studies suggest that the *P. maidis* gut is a dissemination barrier that may prolong viral latency. Bypassing the gut barrier by microinjection significantly reduced viral latency and increased the transmission efficiency. An alternative hypothesis is that introduction of higher virus doses results in higher infection and transmission efficiency. Although we are unaware of the amounts of virus that might be recovered during feeding, our dose-response experiments clearly show that increasing the amounts of injected virus results in higher virus abundance and transmission efficiency in the vector.

4.2. Comparison of MMV dissemination after acquisition by feeding or microinjection

MMV tissue tropism in *P. maidis* after acquisition from maize has

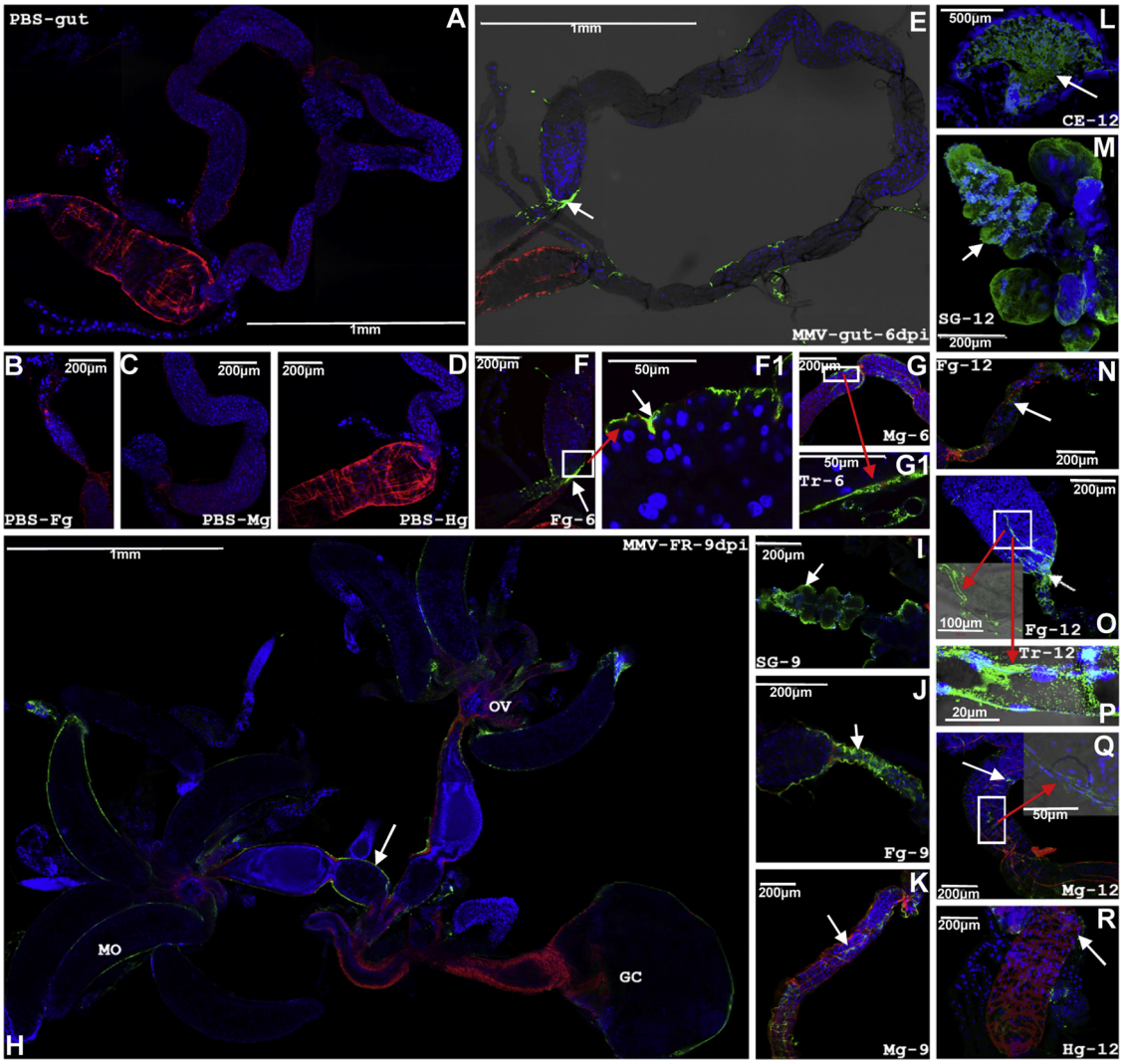

Fig. 6. Progression of MMV infection in *P. maidis* at 6, 9- and 12-days post-injection. MMV presence in the tissues was detected by immunolabeling and confocal fluorescent microscopy. Planthopper organs or tissues were immunolabeled for MMV with Alexa Fluor (green), stained for nuclei with DAPI (blue), and actin was stained with Phalloidin (red). PBS-injected insect tissues, whole gut, foregut, midgut and hindgut tissues are shown in A–D (12-day post-microinjection). Immunolabeled whole gut, foregut, midgut, and trachea MMV-infected insects at 6-day post-microinjection are shown in E–G (See white arrows). MMV-infected foreguts are highlighted in F and F1, and MMV-infected midgut and trachea are highlighted in G and G1, respectively. Labeled MMV-infected female reproduction organs, salivary glands, foreguts and midgut at 9-day post-microinjection are presented in H–K, respectively. At 12-day after injection, MMV-infected compound eyes, salivary glands, foreguts, tracheas, midguts and hindguts are shown in L–R, respectively. FR: Female reproduction organ; Sg: Salivary gland; Fg: Foregut; Mg: Midgut; Hg: Hindgut; Tr: Trachea; MO: Mature oocyte; GC: Genital chamber; OV: Ovary; CE: Compound eyes. Scale bars are shown in each panel, and some MMV-infected tissues (foregut, midgut and trachea) are highlighted with white square and red arrow. Panel L is a cryosection of the insect eye (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

been well described by Ammar and Hogenhout (2008), who found that many tissues support virus infection. Experiments investigating the timing of tissue infections showed that MMV first infected epithelial cells and accumulated in the midgut after a 2-week acquisition access and reached the nerve cord and the compound ganglionic mass by 3 weeks after acquisition. At 4–5 weeks after acquisition, MMV was detected in both the principal and accessory salivary glands and other tissues (Herold and Munz, 1965; Ammar and Hogenhout, 2008). In contrast, our experiments show that microinjected MMV dissemination occurs much more rapidly than *in planta* acquisition. After microinjection, virus was detected in the hemocytes and gut tissues as early as 3 days, and the titer of MMV increased in both tissues from 3 to 12-days post-injection. Microinjection of MMV also led to ˜100% infection of the gut and salivary glands by 9-day post-injection, and the insects exhibited > 90% transmission efficiency. It is important to note that MMV was observed in the injected insect midguts, which suggests a pathway for movement from the hemocoel to the epithelial cells. MMV delivery by microinjection may bypass the *P. maidis* gut barrier but may also result in modulation of dosage responses by modifying defenses of the vector. For example, high doses of injected virus may subvert host immune responses and accelerate virus replication and dissemination. Consequently, delivery of virus by microinjection significantly increases infection and transmission efficiency.

4.3. MMV/dsRNA co-injection for functional genomics characterization

To begin to dissect the molecular interactions between *P. maidis* and MMV, a reproducible and efficient methodology for infecting insects and delivering dsRNA is essential. Delivery of virus by microinjection permits synchronized infections and the ability to adjust amounts of virus delivered. Combining the virus and dsRNA delivery by microinjection also enables unprecedented advancements in virus-host

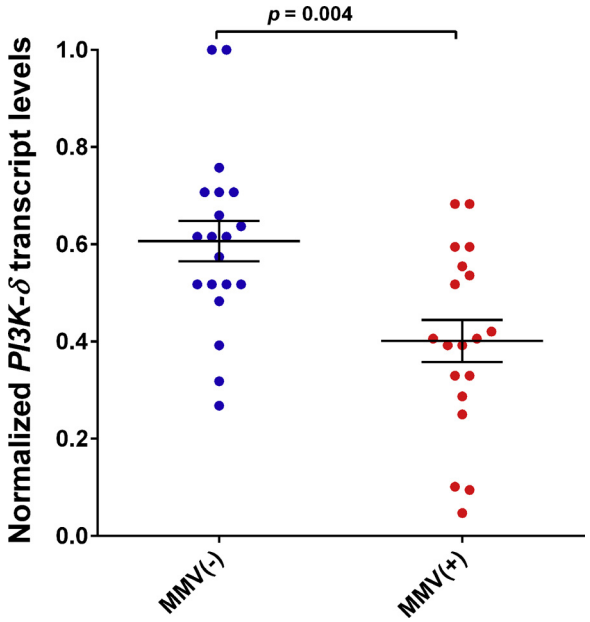

Fig. 7. The normalized abundance of *PI3K-δ* transcripts in MMV-infected [MMV (+)] and non-infected *P. maidis* adults [MMV (-)]. Each point represents an individual insect, and the mean ± standard error of 20 insects is indicated by the horizontal bars. The abundance *PI3K-δ* in MMV-infected insects was significantly lower than that in non-infected insects.

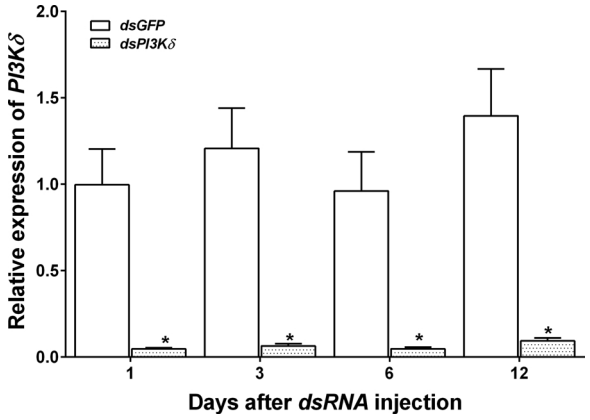

Fig. 8. Expression of the *PI3K-δ* transcript in *P. maidis* after injection of dsRNA. Newly emerged adults (≤ 24 h) of *P. maidis* were injected with 50 nl of dsRNA (200 ng) of *PI3K-δ* or the negative controls of ds*GFP* and H_2O. Insects were harvested every three days for RT-qPCR analysis of *PI3K-δ* gene knockdown. The abundance of *PI3K-δ* was normalized to *P. maidis RPL10* transcripts. Each bar represents the mean and standard error (SE) of the mean of three experimental replicates, each consisting of a group of three insects. The asterisk (*) indicates that the treatment means differ significantly ($p < 0.05$) at each time-point.

interactions, including experiments to knockdown specific genes that may be responsive to virus infection (Martin et al., 2017). Additionally, many basic questions about the *P. maidis*-MMV system remain unanswered including barriers to transmission, mechanisms of cell-to-cell spread, immune system evasion (Sattentau, 2008), and the identity of host cell receptors. In our previous study, we successfully established systemic RNAi in *P. maidis* through feeding and microinjection of dsRNA (Yao et al., 2013). Here, we also developed a high efficiency MMV delivery through microinjection (Figs. 1 and 2), combined with co-injection of dsRNA to facilitate characterization of functions affecting MMV acquisition, replication, movement and transmission.

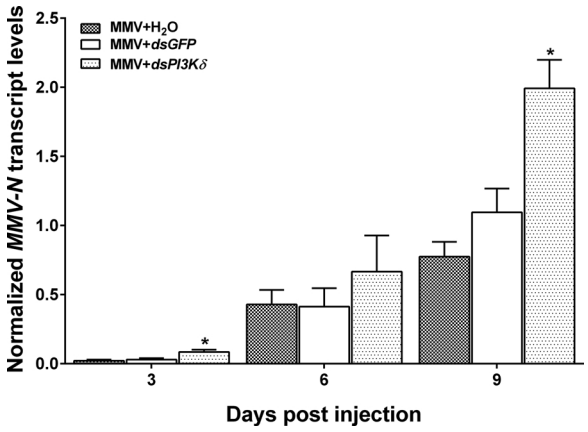

Fig. 9. Normalized *MMV-N* transcript abundance in *P. maidis* after injection with MMV, dsRNA-*PI3K-δ* and dsRNA-*GFP*. Young adult insects were injected with 50 nl mixtures of 5 ng MMV (200 μg/ml) and 100 ng dsRNA-*PI3K-δ* (2 mg/ml), 100 ng dsRNA-*GFP* (2 mg/ml) or H_2O, respectively. Each bar represents the mean and standard error (SE) of the mean of three experimental replicates, each consisting of a group of three insects. The asterisk (*) indicates that the treatments differ significantly ($p < 0.05$) at the indicated time-point.

4.4. PI3Ks/Akt pathway in virus replication

Many viruses appear to activate the PI3K/Akt pathway to maintain infected cell viability and modulate apoptotic signals (Ji and Liu, 2008). Accumulating evidence suggests that viral proteins directly activate PI3K/Akt signaling, and paramyxoviruses, picornaviruses, reoviruses and flaviviruses have been shown to activate PI3K/Akt signaling to forestall apoptosis, drive the cell cycle, and enhance replication, but PI3K/Akt signaling also provides an active immune response component that can be used in cellular defenses against invading viruses (reviewed in Dunn and Connor, 2012). Hence, some viruses, including measles virus, and some rhabdoviruses, bunyaviruses and alphaviruses appear to avoid use of the PI3K/Akt pathway, and some viruses such as vesicular stomatitis virus even inactivate Akt function (Carsillo and Kim, 2010; Dunn and Connor, 2011, 2012). Moreover, PI3K/Akt signaling was actively inhibited by vesicular stomatitis virus replication in mammal cell lines (Dunn et al., 2009).

MMV, as a member of *Rhabdoviridae*, may also avoid or inhibit *P. maidis* PI3K/Akt signaling. Although the basic framework of PI3Ks signaling has been unraveled, the contribution of each PI3K isoform in PI3K/Akt signaling remains unknown in insects. PI3Ks have three classes of isoforms, class I, class II and class III, and the class I of PI3Ks includes p110α, p110β, p110γ, and p110δ (Vanhaesebroeck et al., 2010). In previous work, we found that *P. maidis* midguts infected with MMV perturbed expression of one PI3K/Akt subunit, *PI3Kδ*, (Whitfield et al., 2011), and in this study, we found a subtle, but significant downregulation of *PI3Kδ* in MMV-infected insects. To validate the virus functions and the dsRNA microinjection methodology, we silenced *PI3Kδ* with dsRNA and then measured the virus titer in the planthoppers. The findings reproducibly showed that reducing *PI3Kδ* abundance increased virus accumulation and suggest that this pathway is involved in *P. maidis* responses to MMV infection. Hence, these results imply that MMV might avoid or inhibit PI3K/Akt signaling for replication in *P. maidis*. To date, *PI3Kδ* is one of the best characterized MMV-responsive genes in *P. maidis*, but further studies with specific inhibitors of either PI3K or Akt are necessary to demonstrate involvement of the PI3K-Akt pathway in MMV replication in *P. maidis*.

In summary, virus delivery by microinjection into hemocoel bypassed the insect gut barrier and significantly increased MMV infection and transmission efficiencies. The tissue tropism in MMV microinjected planthoppers was similar to that observed in insects that acquired MMV via *in planta* feeding (Ammar and Hogenhout, 2008); but, microinjected

MMV had much shorter detection and incubation periods in the vector, as well as dramatically shorter times between acquisition and transmission than MMV acquired by feeding on infected plants. This experimental platform will be of significant benefit for functional genomics experiments which eventually should inform strategies for controlling the insect vector and disrupting the virus transmission process.

Author contributions

Yao, J. and Whitfield, A. E. conceived and designed the experiments; Yao, J. performed the experiments; Yao, J., Rotenberg, D. and Whitfield, A. E. analyzed the data; Yao, J. and Whitfield, A. E. wrote the paper..

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jviromet.2019.05.010.

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