Host plant selection and virus transmission by *Rhopalosiphum maidis* are conditioned by potyvirus infection in *Sorghum bicolor*

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Abstract

Many plant viruses are significant pathogens that are able to utilize arthropod vectors to infect a vast range of host plants, resulting in serious economic damage to world food crops. One such crop is *Sorghum bicolor*, grain sorghum, which is the fifth most important global cereal crop, it is grown for human consumption, animal feed, and biofuel. In this study, the *Potyr-viruses* Johnsongrass mosaic virus (JGMV), Maize dwarf mosaic virus (MDMV), Sugarcane mosaic virus (SCMV), and Sorghum mosaic virus (SRMV) were tested for their rates of transmission into tissues of *S. bicolor* by the corn leaf aphid, *Rhopalosiphum maidis*. In addition, virus infected and non-infected *S. bicolor* plants were assessed for their effects on *R. maidis* host plant selection behavior. Further, the propagation of each virus (viral ssRNA copy number) in infected plants was determined using qPCR amplification of viral coating protein gene fragments. The mean rate of JGMV transmission into *S. bicolor* plants by *R. maidis* was significantly lower than transmission of MDMV, SCMV, and/or SRMV. *Sorghum bicolor* plants infected with MDMV, SCMV or SRMV also attract significantly more *R. maidis* than non-infected plants. JGMV-infected plants do not effect a similar change in *R. maidis* plant choice preference. The preference of non-viruliferous *R. maidis* toward *S. bicolor* plants infected with MDMV, SCMV or SRMV, and lack of such attraction by JGMV-infected plants may play a role in virus transmission strategy and efficiency by the vector.

Keywords *Rhopalosiphum maidis* · Potyvirus · Sorghum bicolor · Johnsongrass mosaic virus (JGMV) · Maize dwarf mosaic virus (MDMV) · Sugarcane mosaic virus (SCMV) · Sorghum mosaic virus · Two-choice test · Volatile attraction

Introduction

The earliest fossilized evidence of stylet feeding arthropods was produced roughly 300 million years ago during the Carboniferous period (Labandeira 2013). In the ensuing millenia, intense plant–arthropod interactions have resulted in the evolution of more than 4000 species of phloem-feeding Aphididae (aphids) (Jaouannet et al. 2014), consisting of ~ 100 economically relevant species (Adams et al. 2005) present on the vast majority of the global land-mass (Macfadyen and Kriticos 2012). Due to their global distribution on ~25% of all plants and immense reproductive rates (Dedryver et al. 2010), aphid-related losses to global

agriculture are estimated at \$4.49 billion annually in the U.S. alone (Martin et al. 2015). Aphid feeding on phloem sap reduces chlorophyll and carotenoid, resulting in significantly reduced plant biomass (Riedell and Kieckhefer 1995; Diaz-montano et al. 2007; Macedo et al. 2009; Ni and Quisenberry 2006).

Allelochemical and biophysical traits introgressed into many crop plants have reduced aphid damage (Smith and Chuang 2014). Nonetheless, aphid-related yield losses in U.S. grain sorghum continue to range between 10 and 50% in infested fields (Bowling et al. 2016). The ability of aphids to transmit at least 275 plant viruses further magnifies their detrimental effects on U.S. Agricultural Productivity (1997). Although virus transmission strategies are highly diverse (Dietzgen et al. 2016). Around 75% of all known plant viruses are transmitted via a non-persistent mode of transmission. In this process the virus acquired by feeding on an infected host plant retains in the stylet of the vector without entering other tissues or propagation. The vector remains viruliferous temporarily without horizontal

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or vertical virus transmission (Powell 2005). Among these viruses, the aphid-vectored Potyviridae, is the largest plant virus family, harboring at least 187 members (Adams et al. 2005). Potyviruses have on average a 9.7 kb positive-sense single-stranded RNA genome encoding 10 mature proteins in a single large open-reading frame (Delmas et al. 2019). Molecular studies demonstrated the involvement of these proteins in virus transmission, membrane targeting (including virus movement within plants), viral RNA replication, and virion assembly (Peng et al. 1998; Cronin et al. 1995; Ivanov et al. 2014; Gallo et al. 2018).

Sugarcane mosaic virus (SCMV), Sorghum mosaic virus (SRMV), Maize dwarf mosaic virus (MDMV) and Johnsongrass mosaic virus (JGMV) are members of Potyviridae. All are closely related and arranged in the same phylogenetic clade based on genomic RNA sequence identity, with the exception of JGMV, which is localized in a neighboring branch (Berger et al. 1997; Ward et al. 1992). However, additional phylogenetic analysis on MDMV, SRMV, and SCMV isolates demonstrated a further segregation of these viruses into two groups. On the one hand, a closer related MDMV and SRMV emerged clustering the SCMV virus in a separate clade (Moradi et al. 2017a). These viruses have an overlapping range of host plants that includes sorghum, sugarcane, maize, and Johnsongrass (Seifers et al. 2000; Kannan et al. 2018; Zhang et al. 2016). Plants infected by any of the four viruses develop yellowing leaves with mosaic-like infection patterns (Kannan et al. 2018; Xia et al. 2016; (Grisham and Pan 2007). Potyvirus infections are considered to be the most devastating viral diseases of sugarcane, sorghum, and maize (Moradi et al. 2017b). In addition, maize plants infected with MDMV and SCMV have been shown to suffer reductions in height, weight, and cob weight of 16%, 37%, and 27% respectively (1995).

Recent studies have shown that plant volatile factors play an important role in vector-mediated virus dispersal under natural conditions (Dader et al. 2017). Rice plants infected with Rice Ragged Stunt Virus not only upregulate the expression of genes involved in defense response, but also upregulate those genes involved in volatile-biosynthesis, resulting in plants with increased attraction to vectors, which exponentially promotes their spread of Rice Ragged Stunt Virus (Lu et al. 2016). Similar results were obtained from studies of Cucumber Mosaic Virus, a member of the Bromoviridae virus family, which induce a clear temporal preference of vectors to volatiles emitted from infected plants (Mauck et al. 2010). Other vectors such as the bird-cherry oat aphid, *Rhopalosiphum padi*, and the green peach aphid, *Myzus persicae*, exhibit preferential responses to luteovirus-infected wheat and potato plants compared to uninfected plants. Watermelon mosaic virus (WMV) and zucchini yellow mosaic virus (ZYMV), both Potyviruses, infected plants have been demonstrated to induce the immigration of its

aphid vector (*Aphis gossypii*) towards exposed plant leaves infected with the virus in two-choice experiments (Salvaudon et al. 2013). Those results support the hypothesis that the altered volatile composition of virus-infected host plants is able to manipulate the behavior of aphid vectors (Bosque-Perez and Eigenbrode 2011).

Grain sorghum, *Sorghum bicolor*, is the fifth most important cereal crop in the world and is grown in more than 100 countries to form the basic food staple for more than 500 million people (Luo et al. 2016a). Nevertheless, relatively little is known about the dispersion dynamics and transmission efficiencies of aphids transmitting JGMV, MDMV, SCMV, and SRMV. The goal of this study was to determine the efficiency of *R. maidis* in transmitting each of the four viruses, the effect of *S. bicolor* virus infection on *R. maidis* host plant choice, and viral RNA propagation in *S. bicolor*. Our study resulted in a clear attraction of *R. maidis* towards MDMV, SCMV and SRMV. However, no significant attraction towards JGMV infected sorghum plants was detected. Similarly, high transmission rates were observed for MDMV, SCMV, and SRMV into sorghum plants accompanied by higher absolute viral CP gene accumulation in sorghum plants infected with MDMV, SCMV, and SRMV.

Material and methods

Maintenance and extraction of Potyviruses

Sugarcane mosaic virus (SCMV), Sorghum mosaic virus (SRMV), Maize dwarf mosaic virus (MDMV) and Johnsongrass mosaic virus (JGMV) Potyviruses were obtained in dried and frozen plant tissues from the Kansas State University Agricultural Research Center in Hays, KS, USA. The SRMV isolate was from an unknown location in Kansas. MDMV, JGMV and SCMV were isolated from tissue of plants collected near Hays, KS. Crude plant extracts of each virus were obtained by homogenizing infected plant material in Phosphate buffered saline buffer (PBS: 1.54 mM NaCl, 5.6 mM Na₂HPO₄, 1.1 mM KH₂PO₄, pH 7.4) and centrifuging in 1.5 ml reaction tubes for 10 min at 13,000 rpm in a Thermo Scientific/Legend Micro 21R centrifuge (Fisher Scientific, Pittsburgh, PA, USA). To generate infected plants material for *R. maidis* virus acquisition the clear supernatants containing plant crude extracts, including the individual viruses, were used to brush-inoculate healthy 21- to 28 d-old *S. bicolor* seedlings in the two- to three-leaf stage, each planted in a separate pot. To ensure infection, virus applications were performed twice on 2 consecutive days. Infected plants were kept in 24 °C day:20 °C night and a 14:10 [L:D] h photoperiod illuminated by 32 W fluorescent light source.

Immunological Potyvirus identification

To identify and verify *S. bicolor* plants infections with MDMV, JGMV, SCMV or SCMV ELISA assays were performed 21 d-post-inoculation. In total, 0.05 g leaf tissue from each plant tested plant was homogenized in 200 µl 0.02 M PBST buffer and tested for the presence of each virus with Potyvirus ELISA kits specific for each virus (Agdia Inc., Elkhart, IN, USA). All healthy *S. bicolor* plants were also confirmed negative by ELISA assay. For storage purposes, tissue from positively tested plants was homogenized in 5 ml PBS 10% glycerol buffer, centrifuged at 4000 rpm, and the supernatant stored for later use at -80°C .

Rhopalosiphum maidis virus transmission efficiency

To initiate virus acquisition, approximately 50 adult *R. maidis* obtained from virus-free *S. bicolor* plants were starved for 1 h, placed on leaves of *S. bicolor* infected with one of the tested Potyviruses (JGMV, MDMV, SCMV, and SrMV) and allowed to feed for 30 min, to acquire the virus. For each virus tested, a single viruliferous *R. maidis* was then transferred to 30 non-infected two leaf-stage *S. bicolor* plants using a wet fine brush under a $20\times$ Stereoscope. These aphids were removed manually from exposed plants at 1 h post-transfer and all plants were incubated for 21 d at 24°C day: 20°C night and a 14:10 [L:D] h photoperiod illuminated by 32 W fluorescent light source.

To identify the infection rate a crude cell extract sample obtained from 0.05 g tissue of each plant was subjected to ELISA assay to determine virus transmission rates for each tested Potyvirus according to manufacturer's instructions (Agdia Inc., Elkhart, IN, USA). Each of the four ELISA assays included a negative control obtained from healthy *S. bicolor* plant tissue extract and a commercial positive control. The hydrolysis of the substrate *p*-nitrophenyl phosphate by alkaline phosphatase is catalyzed in the presence of Potyvirus particles turning the testing solution yellowish that can be recorded at 405 nm with a Vmax Kinetic microplate reader (Molecular Devices, San Francisco, CA, USA) after 2 h incubation in darkness at room temperature. Absorption values of $n=30$ independent transmission experiment for each tested Potyvirus were subjected to one-way ANOVA (GraphPad Prism V. 8.20) to determine differences in transmission efficiency of each virus.

Effect of virus infection on *R. maidis* plant preference

For each virus tested, 30 *S. bicolor* plants, each in a separate 10 cm diam $\times$ 9 cm high pot containing Metro-Mix® 360 soil mix (Sun Gro Horticulture Inc, Pine Bluff, AR, USA), were grown under a 24°C day: 20°C night and a 14:10 [L:D]

h photoperiod. Each of the three replicates consisted of 10 two- or three-leaf stage plants being infected with each virus by transferring 5 viruliferous adult *R. maidis* and allowed to feed for 1 h subsequently infecting the plants with one of the tested Potyviruses (JGMV, MDMV, SCMV, and SrMV). After the feeding period *R. maidis* were removed manually. To avoid plant resistance effects induced by aphid feeding on the host plant during the choice assay mock-inoculated control plants treated with 5 adult non-viruliferous *R. maidis* for 1 h were simultaneously generated. Infected and mock-inoculated plants were incubated in separately for 21 d at 24°C : 20°C in a 14 h:10 h light/dark photoperiod illuminated by 32 W fluorescent light source.

A two-choice bioassay was conducted with 21 d post-inoculation plants after confirming healthy and infected plants by ELISA as described above. A 15 cm diameter plastic petri dish with two 1.5 cm diameter glass tubes on opposite sites served as an arena to assess *R. maidis* food source preference. Infected and mock-inoculated control plant leaves were inserted into opposite glass tubes facing each other. The outer end of each tube was plugged with cotton to prevent *R. maidis* escape (Fig. 1). For each preference assay 20 adult and non-viruliferous *R. maidis* were starved for 1 h in a 10 cm Petri dish. Starved aphids were placed by a fine brush in the middle of each arena offering *R. maidis* a choice either between a healthy Sorghum plant or a Sorghum plant infected with one of the four tested Potyviruses. A constant 32 W fluorescent light source maintained a stable illumination across all choice test experiments eliminating a variance in visual cues for *R. maidis* across all experiments. *R. maidis* were allowed to move across the entire arena freely and probe on offered healthy and virus-infected Sorghum plant. After 2 h unrestricted movement the number of individual *R. maidis* located on healthy and virus infected Sorghum plant leaf was counted. The final *R. maidis* counts for settling preference after 2 h was obtained

Fig. 1 Schematic representation of two-choice bioassay system. Leaves of mock-inoculated (a) and potyvirus infected (b) *S. bicolor* plants were inserted into glass tubes on the opposite sites of the arena (c). The arena rests on a cardboard platform (d). For each bioassay 20 adult *R. maidis* starved for 1 h were placed in the middle of the arena and their location recorded after unrestricted movement for 2 h

from 30 independent choice tests. To identify statistically significant differences in *R. maidis* settling behavior between healthy and infected plants the one-way ANOVA (GraphPad Prism V. 8.20) test was performed.

Viral RNA extraction and cDNA synthesis

The abundance of Potyvirus RNA representing the virion particle quantity in plant tissue was quantified with reverse transcription-quantitative PCR (RT-qPCR). For each virus 10 randomly chosen *S. bicolor* plants infected with one of the four tested viruses were used. To obtain total RNA from each plant a Qiagen RNeasy Mini Kit was used following manufacturer's instructions (Qiagen Inc., Germantown, MD, USA). Total RNA was extracted from 0.3 g tissue from each plant. Reverse transcription reactions were performed with 3 µg total RNA as template using the SuperScript III First-Strand Synthesis System (Fisher Scientific, Pittsburgh, PA, USA) and 0.1 µM random hexamers (IDT), according to manufacturer's instructions. The obtained complementary DNA (cDNA) was diluted 1:2, starting from 64-fold dilution, for further processing.

Primer design for reverse transcription-quantitative PCR

Primers suitable to detect MDMV, SCMV, SRMV, and JGMV cDNA were generated from full and partially available viral genomes from NCBI (Table 1). The coat protein (CP) coding regions of each virus were extracted and phylogenetic analyses were performed utilizing MEGA integrated MUSCLE alignment (Edgar 2004) and Neighbour-Joining Tree phylogenetic software (Saitou and Nei 1987) with 500 Bootstrap replications (data not shown). The 21 clustering MDMV, SCMV, and SRMV CP coding regions were realigned and the most highly conserved regions were selected as common primer binding sites for those viruses. All 9 JGMV CP coding regions clustering in a separate

phylogenetic clade were extracted, realigned, and the primer binding sites were determined as previously described for the most conserved gene coding region. The designed degenerated JGMV primer set (5' primer AAGAARGARTAYGAY GTTRATGA, 3' primer: TAYGCTTCWGCKGCRTCA CTRAA), spans a genomic region of 218 bp, whereas the common degenerated primer pair for MDMV, SCMV, and SRMV (5' primer: CAYTTYAGTGATGCAGCTGAAGC, 3' primer: TAYGCTTCWGCKGCRTCACTRAA) covers a distance of 242 bp (Y = C or T, R = A or G, W = A or T, K = G or T).

Reverse transcription-quantitative PCR and standard curve generation

The RT-qPCR analysis was carried out using the CFX Connect Real-Time System and reagents from BIO-RAD, Hercules, CA, USA. Each 20-µl reaction contained 10 µl iTaq Universal SYBR Green Supermix, 1 µl forward and reverse primer, 1 µl cDNA template and 7 µl H₂O. To generate standard curves for each viral CP gene, PCR-amplified coat protein (CP) templates were generated by PCR. The qPCR cycling program was run with the following settings: 3 min initial denaturation at 95 °C and 40 cycles alternating between 30 s at 95 °C, 35 s at 51 °C and 50 s at 72 °C. The amplicons were purified using the Wizard SV Gel and PCR Clean-Up System (Promega) and the DNA concentration for each sample was determined by NanoDrop 2000C (Thermo Scientific) and the DNA copy number calculated as following: $\text{DNA copy number} = (\text{DNA ng } 6.022 \times 10^{23}) / (\text{length } 1 \times 10^9 \text{ 650})$ (Liu et al. 2012). Each sample was diluted in twofold linear fashion. The standard curves for JGMV, MDMV, SCMV, and SRMV CP gene were generated with 1 µl CP gene fragment dilutions as template starting from 1/128 to 1/4096. For each virus and dilution, resulted C_q values were plotted against the log template copy number. The efficiency of the amplification reactions was determined by the linear regression slope of the linear equation and the

Table 1 Potyvirus accessions used for coat protein coding region extraction and comparative analysis to generate suitable qPCR primer sets

Sugarcane mosaic virus	Maize dwarf mosaic virus	Sorghum mosaic virus	Johnsongrass mosaic virus
AY042184	AJ001691	AJ310198	KY952243
AY569692	NC_003377	AJ310196	KY952242
AF494510	AM110758	SMU07219	KY952241
AY149118	FM883211		KY659307
EU091075	JQ403608		KU746868
GU474635	JQ403609		AY387826
NC_003398	JX185302		KR911666
AM110759	JQ280313		JMU07218
AJ278405			Z26920
AJ297628			

regression coefficient (R^2) for each viral CP gene fragment. For CP gene copy number assessment, 10 cDNA samples per tested virus were obtained from infected plants and analyzed for the abundance of viral coat protein cDNA. The reactions were loaded into Hard-Shell PCR Plates (BIO-RAD, catalog# HSP9645) and sealed with Microseal 'B' (BIO-RAD catalog # MSB1001). The qPCR cycling program was run with the following settings: 3 min initial denaturation at 95 °C and 40 cycles alternating between 30 s at 95 °C, 35 s at 51 °C and 50 s at 72 °C. Melt curves were generated on PCR products from 65 to 95 °C in 0.5 °C and 5 s increments. The number of initial template CP copies for each virus was calculated from previously obtained standard curves. The C_q values for each analyzed plant were collected. To identify whether there is a significant differences in CP gene abundance across plants infected with different Potyviruses (JGMV, MDMV, SCMV, and SrMV) the one-way ANOVA (GraphPad Prism V. 8.1.0) test was performed. The actual significant differences in viral CP RNA abundance in plants infected with different viruses was determined by using Tukey test (Lee and Lee 2018) with P values < 0.05 .

Statistical analyses

All datasets were subjected to a one-way ANOVA (GraphPad Prism V 8.1.0) to identify significant changes across datasets (P value ≤ 0.05), a Shapiro–Wilk test (Ghasemi and Zahediasl 2012) for normality and Bartlett's test (Struchalin et al. 2010) for homogeneity of variation were performed. The significance comparison of all possible pairs of means was calculated by Tukey's Honest Significant Difference test. Samples failing the normality test were subjected to Kruskal–Wallis with subsequent Dunn's test means separation test to assess the significance between experimental groups.

Results

Effect of virus infection on *R. maidis* plant preference

In general, *R. maidis* exhibited a significant preference for *S. bicolor* plants infected with MDMV, SCMV or SRMV compared to mock-inoculated control plants by the end of the bioassay period (Fig. 2). The mean numbers of *R. maidis* on plants infected with MDMV, SCMV or SRMV were significantly ($P < 0.05$) greater than those exposed to mock-inoculated *S. bicolor* plants. Although, due to the experimental setup, no direct comparison between *R. maidis* settlement on plants infected with MDMV, SCMV or SRMV is possible, a clearly similar preference towards infected plants is observable. A similar trend can be observed in the mean

Fig. 2 Mean $\pm$ SE number of *R. maidis* on leaves of *S. bicolor* plants infected or mock-inoculated with Potyviruses Johnsongrass mosaic virus (JGMV), Maize dwarf mosaic virus (MDMV), Sugarcane mosaic virus (SCMV), and Sorghum mosaic virus (SRMV) at 2 h after placement in a two-choice bioassay chamber. $n = 30$ infected and 30 mock-inoculated pairs of plants for each virus. Means that do not share a letter differ significantly ($P < 0.05$) using Tukey test

number of *R. maidis* settling on mock-inoculated plants across all conducted choice experiments. However, there is no difference between the mean number of *R. maidis* on JGMV-infected plants and mock-inoculated control plants ($P > 0.05$) (Fig. 2).

Rhopalosiphum maidis virus transmission efficiency

The potential threat posed by the vector *R. maidis* to transmit plant diseases was accessed by testing its ability to transmit Potyviruses (JGMV, MDMV, SCMV, and SrMV) into a suitable host plant such as *S. bicolor*. Therefore, the virus transmission rate by a single viruliferous *R. maidis* into healthy Sorghum plant was measured. The mean virus transmission percentage obtained from 30 individual transmission experiments per virus ranged between 70% for MDMV, 70% SCMV, and 73.33% for SRMV with P value > 0.05 not showing a significant difference between each other. This result suggests a similar *R. maidis* virus transmission potential for those viruses into *S. bicolor*. However, JGMV transmission efficiency by *R. maidis* is with 40% significantly lower if compared to those of MDMV, SCMV or SRMV ($P < 0.05$) indicating a reduced vector-virus or host-virus compatibility (Fig. 3).

ELISA verification of viral transmission

To identify JGMV, MDMV, SCMV and SrMV infected plants on the one hand and to determine the virion quantity qualitatively on the other hand ELISA tests were performed. The one-way ANOVA test computing the ELISA 405 nm absorbance values for plants infected with each virus resulted in P values of 0.4, 0.82, 0.81, and 0.88 for JGMV, MDMV, SCMV, and SRMV, respectively. Absorbance

Fig. 3 *R. maidis* potential to transmit Potyviruses into Sorghum host plant. Mean (±SE) transmission efficiency in percent for Johnsongrass mosaic virus (JGMV), Maize dwarf mosaic virus (MDMV), Sugarcane mosaic virus (SCMV), and Sorghum mosaic virus (SRMV) by a single viruliferous *R. maidis* into *S. bicolor* plants. $n=30$ *S. bicolor* plants. Means that do not share a letter differ significantly ($P<0.05$) due to Dunn's test

values from *S. bicolor* plants infected with MDMV, SCMV, and SRMV yielded mean ± SE ELISA 405 nm absorbance values of 0.71 ± 0.04 , 0.69 ± 0.03 , and 0.71 ± 0.03 , respectively. Those absorbance values correlate to the virion quantity in the tested plant extract. The absorbance data obtained from plant material infected with MDMV, SCMV, and SRMV was not significantly different from one another. In contrast, the mean ± SE ELISA absorbance value of plants infected with JGMV (0.44 ± 0.02) was significantly ($P<0.05$) less than values of plants infected with MDMV, SCMV or SRMV according to Tukey test indicating lower JGMV virion count in host plant *S. bicolor* if compared to other three tested Potyviruses (Fig. 4).

Viral CP gene standard curve generation

The standard curve qPCR slopes of virus specific CP gene amplicons were determined as -3.578 for JGMV, -3.402 for MDMV, -3.2 for SCMV, and -3.359 for SRMV. Those values are within the tolerance of -3.1 and -3.6 representing acceptable amplification efficiency between 90 and 110%. Based on the individual slopes the calculated amplification efficiencies for JGMV, MDMV, SCMV, and SRMV CP gene

fragments are 90%, 90%, 105%, and 98%, respectively. The correlation coefficients (R^2) of all linear regression slopes were >0.99 , ranging from 0.9946 for JGMV to 0.9994 for SRMV confirming a good confidence into the amplification efficiency and subsequently in the absolute quantification of viral particles in plant tissue (Fig. 5).

Viral CP gene copy number quantification

The viral ssRNA genome copy number of each virus was determined based on the C_q values for the viral coat protein (CP) gene in 10 cDNA samples from individual *S. bicolor* plants infected with one of the four tested viruses. Mean ± SE C_q values were as follows: JGMV, 32.4 ± 0.3 ; MDMV, 28.0 ± 0.1 ; SCMV, 26.8 ± 0.3 ; and SRMV, 27.3 ± 0.3 (Fig. 6a) passing the Shapiro–Wilk normality and Tukey test with P values >0.05 (0.59, 0.64, 0.4, and 0.16). The calculated mean JGMV CP gene copy ± SE number of 439 ± 85.1 was significantly ($P<0.001$, $P=0.001$ and $P<0.001$) lower than MDMV, SCMV, and SRMV CP gene copy numbers of

Fig. 4 Qualitative virion quantification by ELISA. Mean (±SE) 405 nm absorbance values of leaf extracts from *S. bicolor* plants infected with Johnsongrass mosaic virus (JGMV), Maize dwarf mosaic virus (MDMV), Sugarcane mosaic virus (SCMV), or Sorghum mosaic virus (SRMV). Means that do not share a letter differ significantly ($P<0.05$) using Tukey test

Fig. 5 Standard curves, PCR amplification plots, linear regression slopes and correlation coefficients of serially diluted coat protein gene fragments from Potyviruses Johnsongrass mosaic virus (JGMV), Maize dwarf mosaic virus (MDMV), Sugarcane mosaic virus (SCMV), and Sorghum mosaic virus (SRMV)

Fig. 6 Mean (±SE) fractional CR cycle (C_q) and mean (±SE) coat protein gene copy in 10 *S. bicolor* plants infected with Potyviruses Johnsongrass mosaic virus (JGMV), Maize dwarf mosaic virus (MDMV), Sugarcane mosaic virus (SCMV), and Sorghum mosaic virus (SRMV). **a** Mean±SE C_q value obtained from 10 *S. bicolor* plants infected with one of the four tested Potyviruses. **b** Calculated mean±SE coat protein gene copy number per ng of total RNA in 10 host plants infected with one of the tested plant viruses. Means that do not share a letter differ significantly ($P < 0.05$) using Tukey test.

8864 ± 691.4 ; 6584 ± 1300 ; and 9065 ± 1500 /ng total RNA. According to Tukey's test multiple comparison test calculated P values ranged between 0.35 and 0.99 for MDMV, SCMV, and SRMV group (Fig. 6b). The Shapiro–Wilk normality test

computing JGMV, MDMV, SCMV, and SRMV coat protein amplicons/ng total RNA template confirmed the normal distribution with P values of 0.16, 0.36, 0.25, and 0.94. Amplicons obtained from qPCR were separated on 2% agarose gel for primer and product quality assessment. DNA bands from 10 plant samples infected with each virus exhibited the expected molecular size of 218 bp for the JGMV CP gene fragment and 242 bp for the closely related MDMV, SCMV, and SRMV CP gene amplicons (Fig. 7).

Discussion

Potyvirus transmission

Potyviruses such as JGMV, MDMV, SCMV, and SRMV cause significant global yield losses in a wide range of crop plants. In this study, inoculation experiments with a single viruliferous *R. maidis* successfully determined the transmission efficiency of each virus into sorghum seedlings. A 1 h *R. maidis* starvation period prior virus acquisition proved to be suitable for maximum virus transmission (Powell et al. 1995). In our transmission efficiency experiments the MDMV, SCMV, and SRMV infection rate varied slightly between 70%–73% was confirmed immunologically by ELISA assay. On the other hand the measured JGMV transmission efficiency rate of 43% was significantly lower. These results are the first to determine JGMV, MDMV, SCMV, and SRMV transmission efficiency by *R. maidis* into *S. bicolor*. However, results of a previous related study (Lucio-Zavaleta et al. 2001) align with the high observed virus transmission efficiencies. *R. maidis*, which also transmits Barley yellow dwarf virus (BYDV), was shown to transmit different BYDV RMV strains at rates ranging from 10 to 82%. Two MDMV haplotypes obtained from Johnsongrass showed a variable transmission rate into maize host plants depending on virus haplotype and transmitting vector. In the same study, *R. maidis* was shown to be the most suitable vector of MDMV into maize among four vectors tested, reaching transmission efficiencies between 29 and 54%, depending on the haplotype, indicating that the abundance of a specific vector population may shape the virus population in a given area (Achon and Serrano 2010).

Previously, *R. maidis* has been shown to transmit SCMV infections into maize seedlings at rates up to 92% (Sahi and Imanat 2003), and similar rates of transmission were observed for MDMV infections into sorghum (Gordon and Thottappilly 2003). A related study with peach potato aphid, *M. persicae*, demonstrated 40% and 85% MDMV transmission into maize seedlings, respectively (Tu and Ford 1971). High coinfection rates with multiple viruses such as SRMV

JGMV **Melt Peaks** **MDMV** **SRMV** **SCMV**

Fig. 7 Agarose gel electrophoresis of Potyviruses Johnsongrass mosaic virus (JGMV), Maize dwarf mosaic virus (MDMV), Sugarcane mosaic virus (SCMV), and Sorghum mosaic virus (SRMV). CP gene amplicons, 100 bp DNA marker and corresponding PCR product melt curves

and SCMV have been also reported. In Tucumán, Mexico, SCMV and SRMV was detected in 70% and 94% of sampled sugarcane with a coinfection rate of 64% (Perera et al. 2012). Analog observations of SRMV and MDMV occurrences reaching 75% in Chinese sugarcane-growing provinces suggest that this Potyvirus maintains a high rate of transmission and co-infection with other Potyviruses (Luo et al. 2016b).

The pairwise analysis of the JGMV CP nucleotide coding sequence to the nucleotide coding sequences of MDMV, SCMV, and SRMV resulted in a slightly greater than 70% identity (de Souza et al. 2017). Thus, considering the essential role of the CP and helper component proteinase (HC-pro) proteins during virus acquisition by *R. maidis* and a higher genetic diversity if compared to other tested viruses might be a factor for the reduced JGMV transmission efficiency by *R. maidis*. Studies on *M. persicae* transmission of the potyvirus zucchini yellow mosaic virus suggest the involvement and necessity of the viral HC-Pro (Peng et al. 1998)], as HC-Pro mutations can result in total loss of infection. Coat protein sequence diversity may also alter the capacity of the vector to acquire the viral helper component (HC-pro), and affect the binding affinity of the CP to the helper protein in the vector stylets (Ng and Perry 2004). On the vector side, CP affinity is determined by HC-pro recognition in the stylet (1987). However, mutations in the HC-pro protein can also affect virus transmission, suggesting a continuous co-evolution and a distinct range of viruses transmitted by the vector with variable efficiencies (Ng and Perry 2004).

Effect of virus infection on *R. maidis* plant preference

Vectors of plant viruses are attracted to infected host plants by visual and olfactory cues that enhance vector attraction, as demonstrated by studies on BYDV- and Cucumber mosaic virus (CMV)-infected plants that induce a clear vector preference compared to uninfected control plants (Ingwell et al. 2012; Mauck et al. 2014). Viral infections cause changes in host plant physiology, that in turn alters the host plant volatile profile. However, to our knowledge, little is known about plant volatile compound content in response to viral infections. Studies of *Cucurbita pepo* plants infected with CMV have shown infected plants to be inferior hosts for the vectors *M. persicae* and *Aphis gossypii* while attracting significantly more vectors by emitting an increased volatile level without significant changes in the overall blend composition causing a winged vector migration to healthy plants after a short probing period. However, in the same study unwinged aphids have shown a significant attraction towards virus infected plants over long period of time (Mauck et al. 2010). Experiments on *M. persicae* attraction towards virus infected potato plants showed a similar effect. Roughly 70%

of tested aphids moved towards infected plants in a dual choice assay and remained arrested over a 1 h testing period (Eigenbrode et al. 2002).

Increased volatile production in wheat infected with BYDV attracts also significantly higher numbers of *R. padi* when plants are in close proximity to one another. Further, headspace volatile analyses of infected plants identified 20 compounds, with (Z)-3-hexenyl acetate being significantly overproduced, suggesting it as a potential *R. padi* arrestant or attractant (Jiménez-Martínez et al. 2004). Similar to those observations, the results of the current study also show the clear appeal of MDMV-, SCMV-, and SRMV-infected plants to *R. maidis*. In contrast, JGMV-infected plants exhibit no such effects on *R. maidis*, and no significant differences in *R. maidis* choice of host plant after 2 h roaming period. Similarly, the majority of alate *R. maidis* has shown a tendency to arrest on infected plants soybean mosaic virus (SMV/Potyvirus) infected soybean plants for more than 1 hour after landing before transitioning to a healthy plant (Feres et al. 1999). The green peach aphid (*M. persicae*) prefers virus infected Jalapeño pepper plants for longer period of time before transitioning to healthy plants (Safari et al. 2019). Studies on host plant quality showed a significant change in soluble metabolites such as sugar and free amino acid content between *Papaya ringspot virus* (Potyvirus) infected and healthy plants and may play a role in aphid host plant choice, growth, and settling behavior (Gadhawe et al. 2019). The preferred settlement and feeding of nonviruliferous vectors on virus infected host plants is a consistently reported observation throughout scientific literature (Mauck et al. 2018).

Viral RNA quantification

The usage of highly specific degenerated primer sets designed based on the viral HC-Pro sequence analysis, including JGMV, for diagnostic purposes has been already reported (Ha et al. 2008). However, no qPCR efficiency for JGMV, MDMV, SCMV, and SRMV CP based degenerated primer sets is reported. Using the designed primer sets for this study the PCR efficiency stretches from 90.2 to 102% with R^2 values between 0.9946 and 0.9994. Considering the fact that MDMV, SCMV, and SRMV standard curve PCR reactions were performed with the same primer sets leads to the conclusion that same the primer pair can be used for diagnostic and quantification purposes for those viruses.

As previously mentioned, the C_q value in real-time PCR analysis resonates the initial template quantity present in the sample. Therefore, a standard curve based absolute quantification of viral ssRNA in plants infected with each of the tested Potyviruses is realizable. The CP copy numbers in

1 ng total RNA from 10 infected JGMV, MDMV, SCMV or SRMV *S. bicolor* plants was determined as following 439.7 for JGMV and 8,886, 6,584, and 9,065 for MDMV, SCMV, and SRMV respectively, confirming the pathological potential towards *S. bicolor*. Nonetheless, the virus propagation rate of JGMV based on CP gene copy number is significantly lower compared to other three tested Potyviruses. Potyviruses belong to secondary movement type viruses recruiting the viral CP protein in addition to other proteins for cell to cell movement (Otulak and Garbaczewska 2011). Decreased pathogen-host plant compatibility might be a factor for the low JGMV CP copy number in *S. bicolor* and affect viral spread throughout plant tissues. Further, evidence for a higher degree of recombination within the Potyviridae group has been found reducing the host specificity and broaden the host plant ranges (Kehoe et al. 2014). Therefore, a thorough bioinformatic recombination event analyses of the JGMV, MDMV, SCMV, and SRMV genomes could provide additional clues about rates of divergent infection and shed light on virus evolution and future pathogenic potential of those viruses.

Conclusions

The results of this study demonstrate that *R. maidis* is an efficient vector of JGMV, MDMV, SCMV, and SRMV into *S. bicolor* able to transmit all four tested Potyviruses. However, its transmission efficiency capacity for JGMV in *S. bicolor* is significantly lower than that for MDMV, SCMV or SRMV. In two-choice bioassays *S. bicolor* infection with MDMV, SCMV, and SRMV manifest in a behavioral manipulation of *R. maidis*, which shows a significant *R. maidis* preference for *S. bicolor* plants infected with MDMV, SCMV, SRMV compared to non-infected or JGMV infected plants. The low propagation of JGMV virus within *S. bicolor* tissue compared to MDMV, SCMV, and SrMV was demonstrated by absolute virion quantification using the qPCR method. The viral CP gene primers designed in this study exhibit a high target specificity and can be used for virus identification or early diagnostics of JGMV, MDMV, SCMV and SrMV virion RNA along with subsequent quantification of virion particles in plant tissue.

However, the reason behind the inferior JGMV transmission efficiency and propagation in *S. bicolor* is still unclear, along with the physiological mechanisms involved in the *R. maidis* attraction towards Potyvirus infected *S. bicolor* plants.

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