GRAPEVINE VEIN-CLEARING VIRUS IS MEALYBUG-BORNE BUT NOT MEALYBUG-TRANSMITTED

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ABSTRACT
This article investigates the ability of two types of mealybugs, citrus (*Planococcus citri*) and longtailed (*Pseudococcus longispinus*) mealybugs, of acquiring and transmitting *Grapevine vein-clearing virus* (GVCV) in a greenhouse setting. Mealybugs are the primary vectors for most Badnaviruses, and only a few species have been shown to be aphid-transmitted. In this study, we tested the acquisition and transmission ability of two mealybug species using GVCV-infected and healthy grapevines in a greenhouse setting for three consecutive seasons. This study determined that acquisition time by the mealybugs could be as low as three days, yet the transmission of GVCV from infected grapevines to healthy grapevines by these two mealybug species was unsuccessful. Additionally, with the use of previously-developed species-specific primers, this study determined that those mealybugs captured in the greenhouse facilities at the University of Missouri could not be identified using these primers, and required primers that were specific to their regional diversity. This study contributes to the wider understanding of the acquisition and transmission of GVCV by certain mealybug species.

Keywords: GVCV, citrus mealybug, longtailed mealybugs, transmission, virus-vector relationships.

فايروس شفافية عروق العنب يحمل بواسطة البق الدقيقي ولكن لا ينقل بواسطته

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المستخلص
أجريت هذه الدراسة لتقصي قدرة نوعين من البق الدقيقي، بق الدقيقي الحمضيات والبق الدقيقي طويل الذيل، على اكتساب ونقل فيروس شفافية عروق العنب (GVCV) في بيئة البيت الزجاجي. البق الدقيقي هو الناقل الأساسي لمعظم الفيروسات التابعة لمجموعة Badnaviruses ، وقد ثبت أن عددًا قليلاً فقط من الأنواع ينتقل عن طريق المنّ. في هذه الدراسة ، اختبرنا قدرة الاكتساب والنقل لنوعين من البق الدقيقي باستخدام أشجار العنب السليمة والمصابة بـ GVCV في بيئة البيت الزجاجي لمدة ثلاث مواسم متتالية. وجدت الدراسة أن وقت اكتساب البق الدقيقي يصل إلى ثلاثة أيام ، ومع ذلك لم ينجح انتقال GVCV من نباتات العنب المصابة إلى السليمة بواسطة هذين النوعين من البق الدقيقي. بالإضافة إلى ذلك ، وجدت الدراسة أن استخدام البواديء المتخصصة والمصممة مسبقًا لا تساعد في تشخيص البق الدقيقي الذي تم جمعه من مناطق البيوت الزجاجية في جامعة ميزوري ، وانما تم تشخيصها باستخدام البواديء التي صممت لتشخيص الانواع المنتشرة في منطقة التنوع الحيوي في وسط الولايات المتحدة. تساهم هذه الدراسة في فهم أوسع لاكتساب ونقل فايروس شفافية عروق العنب GVCV بواسطة أنواع معينة من البق الدقيقي.

الكلمات الرئيسية: GVCV ، بق الدقيقي الحمضيات، البق الدقيقي طويل الذيل، النقل، علاقة الفايروس بالناقل.

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INTRODUCTION

In nature, unlike bacterial, fungal and nematodes phytopathogens, viruses require a vector to spread between hostplants (1, 2, 23, 24). Arthropods are the most common vectors for plant viruses, although fungi and nematodes can also act as vectors (3, 9, 26, 31). While viruses are usually transmitted by a single type of vector, some utilize multiple species of a particular genus. For instance, a virus that is transmitted by aphids will not use other pests for transmissions, such as whiteflies, mites, thrips, fungi, or nematodes (21, 25, 34), yet certain caulimoviruses are transmitted by more than two dozen of aphid species (35). *Grapevine vein-clearing virus* (GVCV; genus: Badnavirus, family: Caulimoviridae) has become an issue in vineyards recently (36, 40). Mealybugs are the primary vectors for most Badnaviruses, yet just a small number have been found to be aphid-transmissible (35). A wide array of Badnaviruses, such as *Citrus yellow mosaic*, *Cacao swollen shoot*, *Piper yellow mottle*, and *Banana streak viruses*, are often transmitted by mealybugs (11, 16, 28, 29, 30, 37). In contrast, only three species within Badnavirus (Spiraea yellow leafspot virus (SYLSV), Rubus yellow net virus (RYNV), and *Gooseberry vein-banding associated virus* (GVBaV) are transmitted by aphids (22). *Banana streak virus* can be vectored by various species of mealybugs (*Planococcus citri*, *P. ficus*, and *Dysmicoccus brevipes*), while *Pseudococcus longispinus* failed to transmit it (32). *Grapevine leafroll-associated viruses* (GLRaV) is a Closterovirus and the causal agent of grapevine leafroll disease, which has been shown to be mealybug-transmissible. Research has shown that several mealybug species, including the longtailed mealybug, grape mealybug, obscure mealybug, and citrus mealybug, can transmit GLRaV with varying degrees of success (17). A recent study showed that grape aphids (*Aphis illinoisensis*) were able to transmit GVCV to Vitis interspecific hybrid cv. Chardonel grapevines in the greenhouse (33). Also, the study provided evidence that aphids transmit GVCV from wild Vitis species to Chardonel, and the wild Vitis serve as a source of GVCV (20, 27, 33). Since GVCV is newly discovered and its biological aspects are yet to be studied, this research investigates (i) the acquisition and transmission of GVCV focused on two species of mealybugs, the citrus mealybug and the longtailed mealybugs, and (ii) whether GVCV can move between infected and healthy grapevines, and (iii) what amount of time is required by mealybugs to acquire GVCV. Additionally, the study utilized existing species-specific primer sets designed to identify mealybug species, and assessed the suitability of those primer sets for amplifying the DNA of two mealybug species collected from Missouri greenhouses.

MATERIALS AND METHODS

Maintaining mealybug populations

The study was conducted over three consecutive years at the University of Missouri-Columbia (MU). Grapevines were acquired for this research through a generous donation from the Qiu laboratory at Missouri State University. Healthy grapevines and grapevines infected with GVCV were also donated and used for this experiment. The infected vines were propagated clonally from an individual GVCV-infected grapevine, identified as LBC0903 (40). The plants were grown and fertilized under greenhouse conditions and were maintained at 4°C during the winter months (December-March). Separate colonies of citrus (*Planococcus citri*) and longtailed (*Pseudococcus longispinus*) mealybugs were collected from two greenhouses at MU. The first was found and collected on cycads in Tucker Greenhouse (population TG) and another on potted grapevines in Ashland Greenhouse (population AG). After collection, both populations were maintained on potato sprouts. Following the inoculation experiments, the potato sprouts were transitioned to a BugDorm (Taichung, Taiwan) to allow for adequate airflow while preventing the mealybugs from infecting healthy plants in the greenhouse (10).

Plant and mealybug DNA extraction

The DNeasy® Plant Mini Kit (Qiagen, Germantown, MD) was utilized for grape leaf DNA extraction, according to the manufacturer. DNA was eluted with 100µl of autoclaved H_2O. Using electrophoresis containing 1% agarose in Tris-Borate EDTA

buffer, the presence of DNA was confirmed and a rough estimate of the DNA concentration was determined. A protocol from Dellaporta et al. (15) was modified for the purposes of this experiment and then used for DNA extraction from the mealybug populations. The mealybugs were put in a 1.5 ml tube and then ground in 400 µl of extraction buffer (100 mM Tris pH=8, 50 mM EDTA pH=8, 500 mM NaCl, 10 mM mercaptoethanol). Then, 27 µl of 20% SDS was added to the tube and mixed, then incubated for 10min at 65℃. Next, a 133µl solution of potassium acetate (5 M) was added to precipitate protein. The solution was then vigorously shaken and incubated on ice for 20 min, then centrifuged at 13,000 rpm for 20 min at room temperature. The supernatant was collected, and the resulting DNA was precipitated by adding 270 µl of isopropanol and then incubated at -20℃ overnight. The pellet was discarded. The following day, the DNA pellet was formed through centrifugation at 13,000 rpm for 20 min, and dissolved in 19 µl H_2O. The DNA quality was assessed through 1% agarose gel electrophoresis. This process was repeated for DNA isolated from the TG and AG populations as a group, as well as for individuals.

Detection of GVCV and mealybugs identification by PCR: To detect GVCV, two sets of primers were utilized: the first set, GVCV2460F (AGACACAGGAGAAAGGGTAAC) and GVCV3122R (GCTAAAACTTTCGAGCTAAC), amplifies a 663 bp segment of the GVCV genome, between 2,460 nt to 3,122 nt; the second set, GVCV4628F (CCATCACTGTACTTGGTCGAC) and GVCV30R (CCCAGATTTGAAACTGGAGCTCTGATACC), amplifies a 3,156 bp segment between 4628 nt and 30 nt. PCR was conducted using the GoTaq® Flexi DNA polymerase (Promega, Madison, WI), following the manufacturer's guidelines. To amplify the DNA from the first primer set, the PCR program consisted of an initial denaturation at 94℃ for 1min followed by 35 amplification cycles (94℃ for 30s, 52.3℃ for 30s, and 72℃ for 1min), and a final elongation at 72℃ for 10min. For the second primer set, the PCR program consisted of an initial denaturation at 95℃ for 5min followed by 35 amplification cycles (95℃ for 30s, 58℃ for 30s, and 72℃ for 3min), and a final elongation at 72℃ for 10min. The quality of the PCR products was assessed using 1% agarose gel electrophoresis.To identify the species of mealybug present in the collected 10 colonies, individual primer sets were used instead of multiplex PCR. For this reason, the PCR method was altered from the protocol developed by Daane et al (14). Here is the list of primers used to identify mealybugs:

1- PCa (TGCAACAATAATTATTGCCATC)
2- PL (CCATTTATCTTTGATCCACAG)
3- PF (CTTTGTTGTAGCTCACTTTCAC)
4- PM (CTGATTTCCTTTATTAATTAATTCA)
5- PC (TAATCTATTTTTATCTATCAATTTAA)
6- PV (ATATTTCTTCTATTGGTTCATTC)
7- FG (GAATCATTAATTTCTAAACGTTTAC)
8- MB-R (CAATGCATATTATTCTGCCATATT)

GVCV acquisition tests

The AG mealybug population was established on the grapevines that were infected with GVCV. The mealybugs fed on the grapevines for one month prior to being tested for long-term GVCV acquisition. At the end of the month, one single leaf was sampled for AG mealybugs, with five to ten mealybugs collected depending on their size, and ground up together. Their DNA was isolated as previously described. To test the short-term acquisition, the GVCV-free mealybugs were used. The mealybugs were placed onto 20 grapevine leaves collected from a vineyard in mid-Missouri (XFC2+F4 Rocheport, Missouri, USA), all of which presented with GVCV symptoms. Ten of the leaves were exposed to mealybugs from the TG mealybugs and the remaining ten leaves were used for mealybugs from the AG mealybugs. Each leaf had five mealybugs, and the leaves were kept in 15 ml tubes. Water was added regularly to maintain the petiole below the water line. The mealybugs fed on the grapevine leaves for a maximum of 5 days before they underwent DNA extraction and isolation. PCR was used

to detect GVCV, according to the previously described procedures.

GVCV transmission test

Members of the AG mealybug colony were introduced to the GVCV-infected grapevines and confined in BugDorms. After approximately one month, the mealybugs had spread throughout the plant. Two GVCV-negative grapevines were introduced to the BugDorm to allow for natural infestation of the mealybugs. It was confirmed that the mealybugs had spread to the healthy grapevines. One month later, Dinotefuran was used to eliminate all remaining mealybugs. DNA was extracted from the healthy leaves and GVCV was detected using the GVCV4628F and GVCV30R primers. The grapevines were transferred to a cooler at 4℃ for the winter months. The PCR test on the grapevine leaves was repeated in the spring of the following year.

RESULTS AND DISCUSSION

Mealybug identification

In order to investigate whether these mealybug populations can acquire and spread GVCV, the TG and AG populations of GVCV-negative colonies, were established on potatoes. Both were collected at the University of Missouri greenhouses. The insects were identified using a method described by (14) and the primers were generated for PCR amplification as mentioned in methodology. For certain species of mealybugs, a band from mealybug DNA can only be amplified using a species-specific forward primer and a universal reverse primer. For this study, the corresponding species-specific forward primer and universal reverse primers were tested individually to determine the species of the mealybugs. Three bands were amplified from both colonies and their nucleotide sequences were determined by cloning them into pGEM-Teasy, a bacterial plasmid (Figure 1). The sequences allowed for the identification of the mealybug in each population, but unfortunately, the primers were not specific enough to identify three species: *P. calceolariae*, *P. longispinus,* and *P. ficus*. The nucleotide sequences, which have been deposited to the genebank and given accession numbers OR033179 and OR083383, showed that the AG mealybug colony contained longtailed and citrus mealybugs. A BLAST search indicated that all the sequences were a strong match for the reference mitochondrial COI gene of *Pseudococcus longispinus* and *P. citri*.

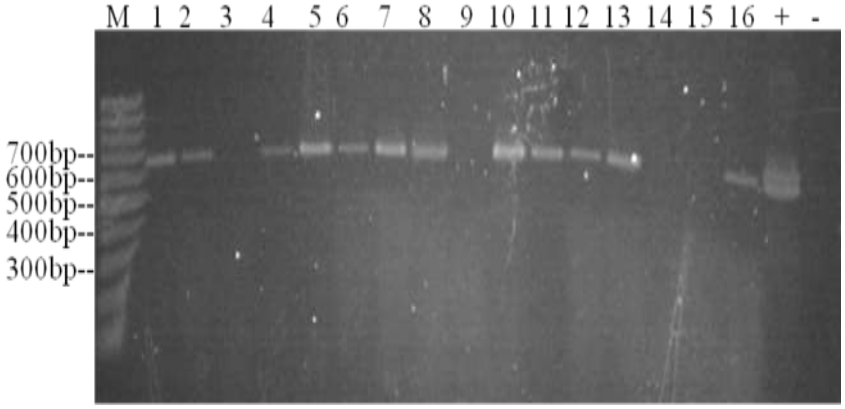

Fig. 1. Amplification of the mealybug DNA region using PCR species-specific primer sets. M: reference DNA ladder (100bp). Lane 1: amplification using Pca and MB-R produced a band of 650 bp. Lane 2: amplification using PL and MB-R resulted in a 600 bp band, Lane 3: amplification using PF and MB-R resulted in a 450 bp band, Lane 4: amplification using PM with MB-R produced a band of 400 bp, Lane 5: PC with MB-R resulted in 350 bp band, Lane 6: amplicon of 250bp produced upon using PV with MB-R, Lane 7: amplification using FG with MB-R produced a band of 150 bp that is not shown in this image that starts from 300bp. Tucker and Ashland-Gravel refer to the source of DNA (mealybug colony) used in each amplification

Mealybugs' acquisition of GVCV

In order to determine the possibility of GVCV acquisition by longtailed and citrus mealybugs, both colonies were moved to infected grapevines. After one month, the AG mealybug colony was established on the grapevines, while the TG colony could not be established. Mealybug DNA isolated from the

AG mealybugs was tested using the GVCV2460F and GVCV3122R primers. Of the 16 samples, 12 were positive for GVCV (Figure 2), demonstrating that members of both species are capable of acquiring GVCV. To better understand the acquisition time for GVCV, members of both AG and TG colonies were placed on detached grapevine leaves that were positive for GVCV.

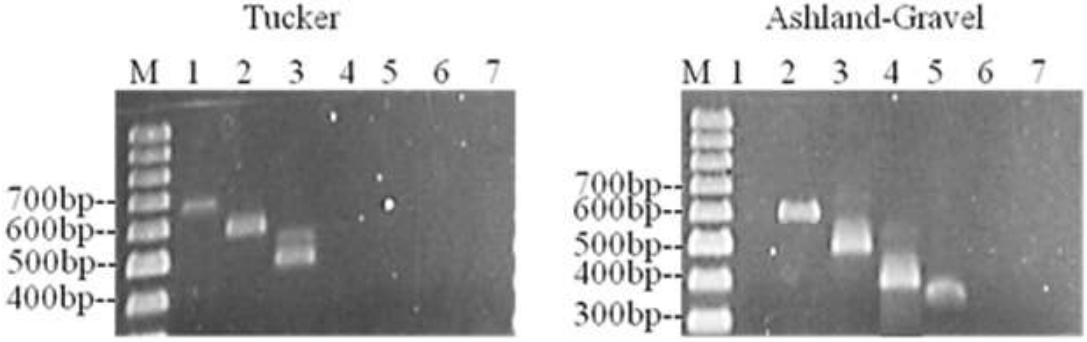

Fig. 2. PCR amplification using primers pair GVCV2460F and GVCV3122R. Results confirm the acquisition of GVCV by mealybug colony from Ashland-Gravel after extended feeding on GVCV-infected grapevines. M: 100bp reference DNA ladder (Fermentas, Pittsburg PA); Lines 1-16: amplifications from DNA of mealybugs collected from leaves of 16 GVCV-infected grapevines; +: positive control (confirmed GVCV-infected leaf); -: negative control, (H2O).

The DNA was extracted and purified from mealybugs after just three days of feeding, at which point GVCV DNA was extracted and amplified from the AG colony (Figure 3). This indicates that the acquisition time for GVCV could be as little as three days. Although individuals in the TG colony were unable to acquire GVCV in the first test, GVCV DNA was amplified from the population in a subsequent test

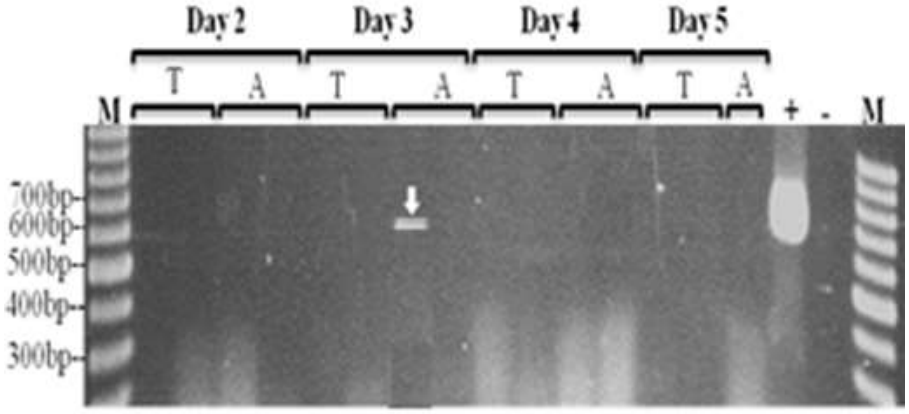

Fig. 3. Acquisition of GVCV by mealybug colony (Ashland-Gravel) from symptomatic grape leaves after feeding for three days. T: DNA purified from Tucker mealybug colony; A: DNA purified from Ashland-Gravel mealybug colony. Extraction of DNA was done, as shown, at different timepoints. Amplification was done using primer set GVCV2460F-GVCV3122R. M: 100bp reference DNA ladder (Fermentas, Pittsburg PA); +: DNA from previously confirmed GVCV-infected grapevine leaf as positive control; -: negative control, (H2O).

GVCV transmission by mealybugs to healthy grapevines: DNA from GVCV was extracted from members of the mealybug colony that fed on grape leaves infected with GVCV, yet transmission to healthy grapevines was still unclear. One month after the AG population was established on infected grapevines, healthy grapevines were introduced to the BugDorms and the population moved into the healthy grapevines. After one month of exposure, the insects were killed using Dinotefuran insecticide. The grapevines were then observed over the course of one year for GVCV symptoms. No GVCV symptoms were observed on any of the grapevines. For PCR analysis, one grapevine leaf was selected for DNA extraction and PCR analysis with the primer pair GVCV4628F and

GVCV30R. At 9 months post-exposure, all plants tested negative for the virus. This particular test was repeated three times, each time with six plants, for a total of 18 plants. All tests resulted in negative PCR analysis for GVCV DNA. The grapevines were placed at 4℃ for the winter months, then transitioned into the greenhouse the following spring. Once again, GVCV symptoms development was monitored, and again, no symptoms were observed. In the summer, two leaves from each of the 18 plants were again tested for GVCV using PCR. Of all 36 samples tested, none tested positive for the virus. The transmission test was repeated the following season with 15 healthy grapevines, which resulted in no symptoms of GVCV or evidence of transmission. It is essential to find new techniques to increase plant yield (4, 5, 6, 7, 8, 12, 25, 38, 39). According to Schoelz and Adhab (35), mealybugs are the prevalent vectors for members of the genus *Badnavirus*. We tested whether longtailed and citrus mealybugs could acquire and transmit GVCV and found that while citrus mealybugs could acquire GVCV, we were unable to demonstrate that they could transmit the virus from infected to healthy grapevines. We encountered difficulties in identifying mealybug species, as the identification of nymphal stages is challenging (13, 18, 19). We attempted to use a modified method (9) for a multiplex PCR with species-specific forward and universal reverse primers. However, as none of the mealybugs in Daane's study were from Missouri, it's reasonable to assume that there might be differences within the mealybug populations in Missouri that are not represented by those primers. Working with GVCV presents difficulties, including the lack of an available GVCV antibody. We determined that those primers that amplify larger fragments were more effective at screening for the virus. Those primers that amplified a 500 bp band (GVCVF1 and GVCVR1) were initially selected for virus screening, yet ghost bands began appearing and the problem could not be rectified, even by re-diluting the primers. A different primer set (GVCV2460F and GVCV3122R), which amplified a 650 bp band, was used instead in the GVCV screening. That set successfully amplified GVCV from samples, without false positives (Figures 2, 3). To avoid any issues with false positives, we then decided to use a third set of primers, GVCV4628F and GVCV30R, which produces an amplicon of approximately 3,000 bp. After this, the contamination problem never occurred again. Grapevine samples were analyzed for GVCV by PCR with the third primer set so as to eliminate any chances of false positives. Because only some GVCV-infected plant organs demonstrate virus symptoms and GVCV is not evenly distributed within infected plant, the asymptomatic leaves from infected plants could test negative for the virus. In GVCV-positive grapevines, the vein-clearing symptoms can often be confused with other disease or heat stress-related symptoms, and because asymptomatic leaves are often mistaken as healthy leaves. Mealybugs of different species exhibit differential virus transmission efficiency. While some species of mealybug have been shown to transmit BSV, others have not (32). In GLRaV experiments, in particular, the transmission ability of different species varies widely, from 19% for longtailed mealybugs to 90% for grape mealybugs (17). In our own experiment, we saw that neither type of mealybug that was used in the experiments was able to transmit GVCV or, at the very least, may have been very inefficient at transmitting the virus. Other mealybug species that were not included in our studies may be capable of transmitting GVCV. The recent finding from Petersen et al. (33) showed that grape aphids act as a transmission vector of GVCV, which supports our hypothesis that mealybugs are not the natural vector of this virus.

Conclusions

This paper demonstrates that particular mealybug species cannot transmit GVCV to healthy grapevines under the conditions applied in this study. To definitively answer this inquiry, more extensive testing should be conducted, including evaluating additional species of mealybugs and increasing the number of plants tested in each trial.

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Conflict of interest statement

The authors declare no conflict of interest.

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