Transmission of *Grapevine leafroll-associated virus 3* by the Vine Mealybug (*Planococcus ficus*)

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ABSTRACT

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Grapevine leafroll disease is caused by grapevine leafroll-associated viruses (GLRaVs). Within this virus complex, GLRaV-3 is the predominant species in the world. Several GLRaVs have been shown to be transmitted from vine to vine by mealybugs although a detailed characterization of transmission biology is lacking. The introduction of the vine mealybug (*Planococcus ficus*) in California and other regions of the world may result in increasing disease incidence of established GLRaVs. We studied the characteristics of GLRaV-3 transmission by the vine mealybug. Our results indicate that the vine mealybug transmits GLRaV-3 in a semipersistent manner. First instars were more efficient vectors than adult mealybugs. GLRaV-3 transmission lacked a latent period in the

vector. Virus transmission occurred with a 1-h acquisition access period (AAP) and peaked with a 24-h AAP. Mealybugs inoculated GLRaV-3 with a 1-h inoculation access period (IAP), and transmission efficiency increased with longer plant access period up to 24 h, after which transmission rate remained constant. After an AAP of 24 h, mealybugs lost GLRaV-3 and infectivity 4 days after virus acquisition. In addition, GLRaV-3 was not transovarially transmitted from infected females to their progeny as detected by reverse transcription polymerase chain reaction. In summary, we systematically analyzed transmission parameters of GLRaV-3 by the vine mealybug and showed that transmission of this virus occurs in a semipersistent manner. This research fills in important gaps in knowledge of leafroll virus transmission, which is critical for development of leafroll disease management practices.

Additional keywords: ampelovirus, closteroviridae.

Grapevine leafroll disease occurs in all major grape-growing areas worldwide and is one of the most destructive viral diseases of grapevines (25,30). At least nine serologically distinct viruses are associated with leafroll disease (1,13). Grapevine leafroll-associated viruses (GLRaVs) are a complex of viruses in the genus *Ampelovirus*, family *Closteroviridae* (24), and GLRaV-3 is the predominant species in the world (5,12,16,26,31,34). GLRaV-2 is also associated with leafroll disease but belongs to another genus, *Closterovirus*, within the same family (13). Members of the genus *Closterovirus* with known vectors are transmitted by aphids in a semipersistent manner (24).

Leafroll disease impacts both vine health and grape quality. The most obvious leafroll symptoms occur in the autumn with leaves developing a strong reddening of the interveinal region in dark-fruited varieties, chlorosis of leaf in white varieties, and downward rolling of leaf margins (18,37). Infection with GLRaVs adversely affects vine growth, berry yield, fruit color, and sugar content of grapes and influences plant phenology through delayed budbreak, flowering, and berry maturity (reviewed in 6). The leafroll disease may cause yield losses of as much as 40% in grapevines (9,30,38). In addition, certain types of graft incompatibility and young vine failure are attributed to infection by these disease agents (15,25). Multiple infections are common in vineyards so more studies are needed to determine the impact of a given GLRaV.

Little is known about the biological and ecological factors that influence the spread and transmission of GLRaVs in vineyards. Until the 1980s, GLRaVs were believed to be only graft-transmissible. Thus, control of grapevine leafroll disease has been traditionally done using clean planting material and roguing of symptomatic plants (14,30). However, in the last two decades, growers and researchers have noticed that the spatial distribution of leafroll disease in vineyards is aggregated, and the prevalence of this disease increases over time (3,5,12,16,17). These observations led researchers to study the possibility of insects acting as vectors of GLRaVs. First evidence of GLRaVs transmission by insects was reported in 1990 (11). Since then, several reports from field and laboratory transmission studies have confirmed the role of mealybugs (Hemiptera: Pseudococcidae) and soft scales (Hemiptera: Coccidae) on the spread of GLRaVs. The mealybugs *Heliococcus bohemicus* and *Phenacoccus aceris* and the soft scale *Parthenolecanium corni* transmit GLRaV-1 under experimental conditions (34). At least eight mealybugs (*Pseudococcus longispinus*, *Pseudococcus viburni*, *Pseudococcus calceolariae*, *Pseudococcus maritimus*, *Planococcus citri*, *Planococcus ficus*, *H. bohemicus*, and *Phenacoccus aceris*) and one soft scale (*Pulvinaria vitis*) have been reported as vectors of GLRaV-3 (2-4,11,15,21,22,26,31,34). *Pseudococcus longispinus* is the only known vector of GLRaV-5 (15).

Despite the importance of these findings for disease control, more work is needed to characterize GLRaVs transmission by insects. Most published studies focused on the identification of mealybug species capable of transmitting specific leafroll viruses (11,31,34). Very few publications quantitatively measured the transmission parameters for mealybug transmission of GLRaVs. Cabaleiro and Segura (4) reported that GLRaV-3 transmission by

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Planococcus citri had some characteristics of semipersistently transmitted viruses because the minimum plant access period for virus acquisition was 3 days and the vector completely lost infectivity 1 h after leaving an infected grapevine. Recently, Cid et al. (7) suggested that transmission of GLRaV-3 by *Planococcus citri* might be circulative according to the presence of the virus particles in the primary salivary glands of its mealybug vector. The clarification of this controversy is important because different vector transmission modes fundamentally affect disease control strategies.

The control of insect-borne plant diseases is often strongly based on a solid understanding of pathogen transmission biology. However, the virus-vector relationships and transmission biology of GLRaVs remain unclear. The objective of our research was to determine transmission parameters for GLRaV-3 by the vine mealybug (*Planococcus ficus*), which has already been shown to transmit GLRaV-3 and *Grapevine virus A* (11). The vine mealybug is an invasive pest in most grape-growing regions worldwide (10), and GLRaV-3 is the most widespread grapevine virus worldwide (25). We suggest using this study as a framework to further examine the transmission biology of other GLRaVs by insect vectors.

MATERIALS AND METHODS

Insects, virus, and plants. We established a vine mealybug colony, which was derived from field-collected material in vineyards near Del Rey, California, on butternut squash (*Cucurbita moschata*) in a growth chamber at $22 \pm 2^\circ\text{C}$. First instars of vine mealybugs were used in all experiments except where indicated. Virus source and healthy test grapevines (*Vitis vinifera*) were obtained from the Foundation Plant Services (FPS) at the University of California, Davis. LR101 (FPS virus accession number) and LR109 were used in this research. LR101 source vine (cv. Italia-3) was infected with GLRaV-3 only, and LR109 source vine (cv. Pinot Noir) was infected with GLRaV-2, GLRaV-3, GLRaV-7, *Grapevine rupestris stem pitting-associated virus*, and *Grapevine fleck virus*. Source vines used for acquisition feeding were rooted from dormant cuttings and grown in a greenhouse until they reached about 60-cm tall. We tested all virus source plants by reverse transcription polymerase chain reaction (RT-PCR, described later) to ensure they were GLRaV-3 infected. Healthy test plants used for mealybug transmission tests were rooted from dormant cuttings of grapevine cv. Cabernet Franc and grown in a greenhouse until they reached about 15-cm tall with approximately four expanded leaves. Cabernet Franc is frequently used as a biological indicator for leafroll disease because of its high susceptibility to the pathogen (30). All experiments had greenhouse negative controls, grapevines from the same batch of test plants that were not exposed to mealybug vectors. None of the controls was positive for GLRaV-3.

Effect of mealybug life stage on virus transmission. We assayed adult and first instar mealybugs for virus transmission efficiency. Immediately before the experiment, source vine cuttings (30 cm) were cut from GLRaV-3-infected plants and maintained in flasks of water. Then we transferred adult mealybugs from colonies to the leaves of source cuttings using a soft brush. After adults had a virus acquisition access period (AAP) of 24 h, we individually transferred mealybugs with the brush from the source cuttings to test plants. Five adults were transferred to each test plant and caged. Clip cages were made using a plastic cup (25-mm diameter $\times$ 25-mm height), flexible metal hair clip, and foam tape to seal the gap between the cage and leaf surface. We placed the cage on the upper surface of leaves of test plants. First instar mealybugs were allowed to move onto 30-cm source vine cuttings laid on mealybug colonies. Two hours afterward, we removed the source cuttings from the mealybug colonies and maintained them in flasks of water. After the first instars were

given an AAP of 24 h, we gently shook them off the source tissue onto 0.5-cm diameter paper disks. Approximately five mealybugs on a paper disk were then transferred to each test plant and caged on the leaf surface. After a virus inoculation access period (IAP) of 24 h, we removed adults or first instars from the test plants and then treated these plants with an insecticide (Safari) to kill all mealybugs. Twenty-five test plants were inoculated by adults or first instars, respectively, under these conditions. We performed this experiment twice, once each using leafroll stock LR101 or LR109 as virus sources. Test plants were maintained in a greenhouse and sprayed regularly with insecticide and fungicide until the RT-PCR assay. All plants were periodically pruned to avoid overgrowth. Transmission efficiency was analyzed by χ^2 test between two life stages and between two leafroll stocks.

Effect of plant access period on virus transmission. We investigated the effect of the plant access period on GLRaV-3 transmission efficiency by mealybugs. Using the methods described previously, first instar mealybugs were allowed to move from colonies to LR101 source vine cuttings. After an AAP of 1, 2, 4, 8, 24, or 48 h on LR101 source cuttings, we transferred mealybugs with paper disks from the source tissue to test plants. Five mealybugs were transferred to each test plant and caged on the leaf surface. We inoculated seven replicate test plants at each AAP. After an IAP of 48 h, we removed mealybugs from the test plants and then treated these plants with an insecticide to kill all mealybugs.

A similar experiment was conducted to determine the effect of plant access period on GLRaV-3 inoculation efficiency by mealybugs. After an AAP of 48 h on LR101 source vine cuttings, we transferred first instars with paper disks to test plants. Five mealybugs were transferred to each test plant and caged on the leaf surface. We inoculated seven replicate test plants at each IAP. After an IAP of 1, 2, 4, 8, 24, or 48 h, we removed mealybugs from the test plants, and then treated these plants with an insecticide. We performed both AAP and IAP experiments twice, for a total of 14 replicates at each AAP or IAP. Test vines maintenance and pesticide spray were the same as described previously. Effects of AAP and IAP on GLRaV-3 transmission rates were tested using separate logistic regressions (8). For these tests, AAP and IAP were log-transformed to minimize residual deviance. The potential for differences between the two trials was tested by including trial as a blocking factor.

Latent period for vector transmission. To verify whether mealybug transmission of GLRaV-3 requires a latent period, experiments were carried out with short AAP and IAP. First instar mealybugs were allowed to move from colonies to LR101 source vine cuttings. After an AAP of 1 or 4 h, we transferred mealybugs from the source cuttings to test plants. Fifty mealybugs were transferred to each test plant and caged on the leaf surface. Groups of 50 mealybugs were used for virus inoculation to account for the expected low transmission efficiency when using short AAP and IAP. Mealybugs with a 1-h AAP were allowed to feed on the test plants for a 1-h IAP; mealybugs with a 4-h AAP were allowed to feed on the test plants for a 4-h IAP. After the IAP, we removed mealybugs from the test plants and treated those with an insecticide. We inoculated 10 replicate test plants for each AAP-IAP combination. We performed this experiment three times. Test vines maintenance and pesticide spray were the same as described previously.

Virus retention and persistence of infectivity. To determine the period that mealybugs retain GLRaV-3 and remain infective, the following experiments were performed. First instar mealybugs were allowed to move from colonies to LR101 source vine cuttings. After an AAP of 24 h, we shook the mealybugs off the source tissue onto potato sprouts and butternut squash for post-acquisition feeding. Potato and squash are nonhosts of GLRaV-3 (23). Ten mealybugs were transferred to each test plant and caged on the leaf surface daily after transferring onto potato sprouts and

butternut squash. We inoculated test plants with mealybugs after 0, 1, 2, 3, 4, 5, or 6 days on the nonhosts. After an IAP of 24 h, we removed mealybugs from the test plants and treated them with an insecticide. We performed this experiment twice. To determine how long mealybugs retained GLRaV-3 after the AAP, we randomly sampled 20 individuals for the presence of GLRaV-3 by immunocapture RT-PCR (described later) after 0, 2, and 4 days on the nonhosts. We performed this detection experiment three times and sampled the same batches of mealybugs used for virus inoculation. Test plants maintenance and pesticide spray were the same as described previously. We analyzed the effects of post-acquisition feeding period on GLRaV-3 transmission to plants and GLRaV-3 retention in insects using separate logistic regressions.

Transovarial passage. We examined whether female mealybugs transmit GLRaV-3 vertically to their progeny. We put a squash that was colonized with mealybugs next to an LR101 source vine, so mealybug nymphs were able to move from the squash to the source vine. Two weeks later, we checked daily for mealybugs feeding on the source vine to collect females and the egg sacs they produced. Five females and their egg sacs were collected for RNA extraction during each trial. The presence of GLRaV-3 in each female and egg sac was detected by RT-PCR (described later). We performed this experiment two times.

Virus detection. For all experiments, we assayed test plants for GLRaV-3 infection using a modified version of an RT-PCR procedure optimized for grapevine tissues (29). At least three leaf petioles from each test plant were collected and combined for RNA extraction to avoid possible problems with uneven distribution of the virus within plants. RT-PCR was carried out with the OneStep RT-PCR kit (Qiagen, Valencia, CA). The reverse transcription was performed at 50°C for 30 min, followed by a PCR activation step at 95°C for 15 min, amplification of 35 cycles at 94°C for 30 s, 56°C for 30 s, and 72°C for 1 min, and a final extension step at 72°C for 10 min. We analyzed amplification products by electrophoresis on 1% agarose gels and visualized on a UV transilluminator following ethidium bromide staining. Single 546-bp band on the gel was expected for virus-infected plants (29).

We inoculated seven test plants for three trials (21 plants in total) with GLRaV-3 by groups of five mealybugs per plant (48-h AAP and 48-h IAP) and monitored the numbers of plants positive for GLRaV-3 by RT-PCR 2, 4, 6, and 8 months after inoculation. We found GLRaV-3 was readily detected in the test plants 2 months after inoculation and the proportion of GLRaV-3 positive plants was constant from 2 to 8 months. Therefore, we decided to assay all transmission experiments by RT-PCR 4 months after inoculation.

We detected the presence of GLRaV-3 in each individual mealybug by RT-PCR using an immunocapture protocol modified from Rowhani et al. (33). Antiserum of GLRaV-3 was kindly provided by Adib Rowhani (University of California, Davis). Briefly, each mealybug was macerated in 25 µl of enzyme-linked immunosorbent assay (ELISA) extraction buffer (500 mM Tris-Cl, 140 mM NaCl, 2% PVP-40, and 0.05% Tween 20, pH 8.3) under a dissecting microscope. PCR tubes were precoated with

25 µl of GLRaV-3 antiserum for 60 min at 37°C. After three washes of phosphate-buffered saline (PBS)-Tween, we placed each whole insect extract into individual precoated tubes, and then incubated the tubes for 60 min at 37°C followed by three washes of PBS-Tween. RT-PCR was carried out with the same reagents and conditions described previously. PCR amplification was adjusted to 40 cycles in order to detect the low virus titer in single mealybug samples. We analyzed amplification products by gel electrophoresis as described above.

We extracted total RNA from individual females and egg sacs using TRIzol (Invitrogen, Carlsbad, CA). Individual females were macerated in 100 µl of TRIzol. Eggs in an egg sac were macerated in 10 µl of ELISA extraction buffer under a dissecting microscope before adding 100 µl of TRIzol. The subsequent phase separation and RNA precipitation followed the manufacturer's instruction. The presence of GLRaV-3 in each female and egg sac was detected by RT-PCR with the same reagents and conditions described previously, except total RNA was used as templates. PCR amplification was adjusted to 40 cycles to detect the low amount of viruses. We analyzed amplification products by gel electrophoresis as described previously.

RESULTS

Effect of mealybug life stage on virus transmission. The effect of mealybug life stage on GLRaV-3 transmission was significant (χ^2 test, $P < 0.001$ [LR101], $P = 0.003$ [LR109], Table 1). There was no significant difference between the transmission results for LR101 and LR109 stocks (χ^2 test, $P = 0.5$). First instar mealybugs were capable of transmitting GLRaV-3 from source vines to test plants when groups of five mealybugs were allowed to feed for 24-h virus acquisition and 24-h inoculation. Adult mealybugs did not transmit GLRaV-3 from LR101 but transmitted GLRaV-3 from LR109 with very low efficiency (1 out of 25 test plants).

Effect of plant access period on virus transmission. The effect of plant access period on GLRaV-3 acquisition and inoculation efficiencies was determined with mealybug transmission assays. The transmission rates at different AAP and IAP are shown in Figure 1. Virus acquisition occurred with a 1-h AAP and reached the maximum with a 24-h AAP when groups of five mealybugs were used for virus inoculation with a 48-h IAP (Fig. 1). We pooled the data from the two trials and analyzed them by

Fig. 1. Transmission rate of *Grapevine leafroll-associated virus 3* for various acquisition and inoculation access periods. Filled diamonds indicate the transmission rates following various acquisition access period (AAP) and a 48-h inoculation access period (IAP). Open circles indicate the transmission rates following a 48-h AAP and various IAP. Transmission tests were carried out with groups of five first instar mealybugs per plant.

TABLE 1. Transmission efficiency of *Grapevine leafroll-associated virus 3* (GLRaV-3) by groups of five mealybugs per plant

	No. of infective plants				
	First instar	P _s ^a	Adult	P _s	P ^b
LR101	16/25 ^c	0.18	0/25	0	<0.001
LR109	11/25	0.11	1/25	0.01	0.003

^a P_s: Swallow estimator, probability that a single mealybug transmits GLRaV-3.

^b Significance of transmission efficiency difference between first instars and adults (χ^2 test).

^c Number of plants positive for GLRaV-3/number of plants tested.

logistic regression because there was no significant difference of variance between two trials. There was a significant and positive effect of AAP on virus transmission rate ($z = 2.59$, $P = 0.010$, $df = 10$).

After an AAP of 48 h, groups of five mealybugs inoculated GLRaV-3 to grapevines with a 1-h IAP (Fig. 1). Transmission rates increased with longer plant access periods and reached the maximum with a 24-h IAP. We pooled the data from the two trials and analyzed them by logistic regression because there was no significant difference of variance between two trials. There was a significant and positive effect of IAP on virus transmission rate ($z = 3.53$, $P < 0.001$, $df = 10$). Maximum transmission efficiency occurred at 24 h of AAP and IAP as expected for semipersistently transmitted viruses.

Latent period for vector transmission. We tested whether mealybugs inoculate GLRaV-3 immediately after they acquire the virus. Virus transmission occurred with as short as a 1-h AAP followed by a 1-h IAP when groups of 50 mealybugs were used for virus inoculation (Fig. 2). A short period between virus acqui-

sition and inoculation is consistent with the hypothesis that GLRaV-3 does not circulate within its vector before transmission occurs.

Virus retention and persistence of infectivity. Immuno-capture RT-PCR analysis of mealybugs having various post-acquisition feeding periods showed that after a 24-h AAP, mealybugs retained GLRaV-3 up to 2 days (Fig. 3). According to our transmission experiments, mealybugs lost their infectivity 4 days after the end of virus acquisition (Fig. 3). We pooled the data from the trials and analyzed them by logistic regression because there was no significant difference of variance between the two trials. There was a significant and negative effect of postacquisition period on virus transmission rate ($z = -3.84$, $P < 0.001$, $df = 11$) and GLRaV-3 positive mealybugs ($z = -3.70$, $P < 0.001$, $df = 7$). In addition, the virus transmission efficiency was positively correlated to the proportion of GLRaV-3 carrying mealybugs ($r^2 = 0.95$, $P < 0.0208$, $df = 1$). This relationship was not significant probably because only three time points were available for the correlation analysis. The results imply that mealybugs lose GLRaV-3 and infectivity within 4 days postacquisition feeding.

Transovarial passage. We tested the presence of GLRaV-3 in female mealybugs and the egg sacs they produced by RT-PCR. All 10 females were positive for GLRaV-3 whereas none of their egg sacs was positive for this virus. This result suggests that GLRaV-3 is likely not transovarially transmitted from infected females to their progeny, although we cannot rule out the possibility that the virus titer in eggs was too low to be detected by RT-PCR.

DISCUSSION

We systematically analyzed the transmission parameters of GLRaV-3 by the vine mealybug. Our results indicate that the vine mealybug transmits GLRaV-3 in a semipersistent manner. Virus transmission occurred with a 1-h AAP and a 1-h IAP, and transmission efficiency increased with a longer plant access period up to 24 h. GLRaV-3 transmission lacked a latent period in the vector. Mealybugs lost GLRaV-3 and infectivity 4 days after virus acquisition. This information will be valuable for the development of leafroll disease management practices.

Vector life stage is an important factor for the transmission of many insect-borne plant viruses (27). Our results indicate that first instars of the vine mealybug are more efficient vectors than adult mealybugs. However, we do not rule out the possibility that

Fig. 2. Transmission rate of *Grapevine leafroll-associated virus 3* for two sequential acquisition access period (AAP) and inoculation access period (IAP); vertical bars indicate standard error. Transmission tests were done using groups of 50 first instar mealybugs per plant.

Fig. 3. Transmission rate and virus retention of *Grapevine leafroll-associated virus 3* (GLRaV-3) by mealybug nymphs. Dark columns indicate the transmission rates of GLRaV-3 determined by reverse transcription-polymerase chain reaction (RT-PCR) 4 months after virus inoculation. White columns indicate the proportion of GLRaV-3 carrying mealybugs after various postacquisition feeding periods (0, 2, and 4 days). Vertical bars indicate standard error. Transmission tests were done using groups of 10 first instar mealybugs per plant following a 24-h acquisition access period and a 24-h inoculation access period. Numbers above columns indicate the number of positive plants and the number of tested plants. nd = not determined.

our experimental methodology, which included physically transferring mealybugs, may have affected the behavior of the more sessile adult mealybugs, as compared with the more mobile first instars. Petersen and Charles (31) reported that first instars of *Pseudococcus longispinus* and *Pseudococcus calceolariae* transmitted GLRaV-3, but not third instars. Because first instars are the most mobile stage of mealybugs, and windborne dispersal of first instars is much easier than that of later instars and adults, the spread of GLRaV-3 under field conditions may be largely driven by this life stage. If that is the case, the spread of grapevine leafroll disease will occur primarily when first instars are present in large numbers in vineyards (mealybug species dependent), narrowing the time window for pathogen dispersal to specific periods of a grape-growing season.

We calculated the probability that a single mealybug transmitted GLRaV-3 using the estimator proposed by Swallow (35). Using five insects per test plant with a 24-h AAP and a 24-h IAP, the Swallow estimator (P_s) was 0.11 to 0.18 for first instars and 0 to 0.01 for adults (Table 1). These data are much higher than the Swallow estimator of GLRaV-3 transmission by groups of five *Planococcus citri* nymphs with a 3-day AAP and a 15-day IAP ($P_s = 0.02$) (4). The comparison suggests that the vine mealybug might be a more efficient vector of GLRaV-3 than *Planococcus citri*, although parallel transmission studies with different mealybug species are needed to reach a definitive conclusion. Such comparative studies will be of great importance to determine the relationship between vector species and the potential for leafroll disease spread under different field conditions.

So far, all members of the family *Closteroviridae*, including the genera *Ampelovirus*, *Closterovirus*, and *Crinivirus*, with known vectors are transmitted in a semipersistent mode (24,28). Mealybugs also transmit other plant viruses semipersistently (e.g., *Banana streak virus* and *Cocoa swollen shoot virus* [both are in the family *Caulimoviridae*]) (20,32). Plant viruses transmitted semipersistently are, in general, acquired by vectors within minutes/hours of plant access. After acquisition vectors retain viruses and remain infective for hours/days. All our transmission data indicate that the vine mealybug transmits GLRaV-3 in a semipersistent manner. Mealybugs acquired and inoculated GLRaV-3 with a 1-h AAP and a 1-h IAP, respectively, and transmission efficiency increased with longer plant access periods up to 24 h. We also found the vine mealybug transmitted GLRaV-3 within 2 h from virus acquisition to inoculation. This period included the time needed for mealybugs to settle down and start feeding, so the actual latent period, if any, was shorter than 2 h. The vine mealybug lost GLRaV-3 and infectivity 4 days after leaving an infected grapevine. The short virus retention time in mealybugs implies GLRaV-3 does not circulate or replicate within its vector, a characteristic of semipersistent transmission (27, 28). In addition, GLRaV-3 was likely not transovarially transmitted from infected females to their progeny. Only two publications reported the temporal characteristics for mealybug transmission of GLRaV-3, and the information was very limited (4,15). The minimum plant access period for GLRaV-3 acquisition by *Planococcus citri* was 3 days, and *Planococcus citri* lost infectivity 1 h after leaving an infected grapevine (4). *Pseudococcus longispinus* inoculated GLRaV-3 within 24 h of plant access period (15). Their results are also consistent with the characteristics of semipersistent transmission. Recently, Cid et al. (7) suggested that transmission of GLRaV-3 by *Planococcus citri* might be circulative based on the presence of virus particles in the primary salivary glands of its mealybug vector. Results from our study do not support the hypothesis for circulative transmission but provide data that GLRaV-3 is transmitted in a semipersistent manner. Nault (27) concluded that the mode of plant virus transmission is generally the same for a given virus even if it is transmitted by various insect vectors. We suggest that, even though GLRaV-3 particles may circulate into the hemocoel, this is

not relevant for transmission, as already observed for some beetle transmitted viruses (36).

There are many diseases constantly threatening the grape industry worldwide (25). These diseases include new emerging diseases as well as the epidemic reemergence of previously established diseases that were rare or of little importance. An example is the reemergence of Pierce's disease as a major production limiting factor in California after the introduction of the glassy-winged sharpshooter into the state (19). The vine mealybug was accidentally introduced into southern California in the early 1990s (10). The invasion of the vine mealybug in California and elsewhere may result in increased disease incidence of established GLRaV-3. However, there are other grape-associated mealybug species already established in California and those may also be driving current epidemics in northern regions of the state. Determining the role of various vector species on the spread of leafroll disease is of great importance; yet, other factors such as virus strain—grape varietal interactions should also be investigated as potentially important components of GLRaVs epidemics.

When mealybugs were first determined as potential vectors of grapevine viruses, transmission and epidemiology studies were hindered by limited tools to (i) identify the viruses they transmit and (ii) detect low virus titers in plants and mealybugs. The recent improvements in molecular detection (e.g., RT-PCR and real-time quantification) provide better tools to study the transmission biology of GLRaVs. This study provides a framework for future studies on GLRaVs transmission by different mealybug and coccid vectors, and the garnered information will be used to develop better management practices for grapevine leafroll disease.

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