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Ferrisia gilli (Hemiptera: Pseudococcidae) Transmits Grapevine Leafroll-Associated Viruses

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Abstract

Several mealybug species are vectors of grapevine leafroll-associated viruses (GLRaV), which cause the economically important grapevine leafroll disease in grape-producing regions worldwide. The mealybug *Ferrisia gilli* Gullan is a new pest of grapevines in El Dorado County, located in the Sierra Foothill wine-growing region of California. GLRaV species 1, 2, 3, and 4LV have been detected in vineyards with symptomatic vines in the Sierra Foothills. We conducted controlled virus acquisition and transmission experiments using source vine accessions infected with different combinations of GLRaV. We determined that *F. gilli* acquired GLRaV 1, 2, 3, and 4LV, and transmitted GLRaV-3 and GLRaV-4LV to uninfected recipient vines. Like numerous other mealybug species, in addition to causing direct damage to vines, *F. gilli* poses a threat to the grape industry as a vector of economically damaging viruses.

Key words: Closteroviridae, *Ferrisia gilli*, grapevine leafroll disease, mealybug, vector

Grapevine leafroll disease (GLD) is an economically damaging disease caused by a complex of virus species in the family Closteroviridae (Martelli et al. 2002), which are present in most commercial grape-growing regions (Almeida et al. 2013). GLD symptoms include leaf reddening with green venation (prominent in the fall season in red-berried cultivars), downward rolling of leaf margins, phloem disruption, yield reduction, shortened vineyard life span, and adverse impacts on wine quality resulting from decreased sugar accumulation and delayed maturation (Over de Linden and Chamberlain 1970). Virus species causing GLD were previously named *Grapevine leafroll-associated virus 1*, *Grapevine leafroll-associated virus 2*, and so on sequentially (GLRaV-1, GLRaV-2, GLRaV-n; Martelli et al. 2002), but were recently condensed into GLRaV-1, -2, -3, -4LV (LV = like viruses), and GLRaV-7, due to a lack of support for sufficient genetic differentiation among virus isolates that were initially classified as different species (Maree et al. 2013). GLRaV-1, -3, and -4LV are in the genus *Ampelovirus*, and are transmitted by mealybugs (Tsai et al. 2010, Le Maguet et al. 2012, Blaisdell et al. 2015). GLRaV-2 is in the genus *Closterovirus*, and has no currently known vector. Finally, GLRaV-7 is in the genus *Velarivirus*, also has no known vector, and may not cause disease symptoms (Al Rwahnih et al. 2012, Reynard et al. 2015). With respect to GLD, GLRaV-3 is the most common and important species in the complex worldwide (Almeida et al. 2013, Maree et al. 2013).

Plant to plant transmission of GLRaV-3 by the mealybug *Planococcus ficus* (Signoret) provided the first demonstration of an insect vector of a GLD pathogen (Engelbrecht and Kasdorf 1990). Mealybug species that transmit GLRaV include *Pseudococcus maritimus* Ehrhorn, *Ps. viburni* (Signoret), *Ps. longispinus* (Targioni-Tozzeti), *Ps. calceolariae* (Maskell), *Ps. comstocki* (Kuwana), *Planococcus citri* (Risso), *Phenacoccus aceris* Signoret, and *Heliococcus bohemicus* Sulc (Daane et al. 2012, Almeida et al. 2013). The soft scales *Pulvinaria vitis* (L.) and *Parthenolecanium corni* (Bouché) are also vectors (Hommay et al. 2008, Bahder et al. 2013). Impressive here is the breadth of mealybug and soft scale species that transmit the *Ampelovirus* GLRaV species. The correct identification of insect vectors is essential for the development of effective disease management practices. Still, Tsai et al. (2010) proposed that there is no evidence of virus–vector species specificity for transmission, and to date it appears that *Ampelovirus* GLRaV species can be transmitted by all grape-associated mealybug species tested. Therefore, all mealybugs colonizing grapevines should be considered as potential vectors of GLRaV. However, transmission efficiency can differ greatly among mealybug species (e.g., Blaisdell et al. 2015).

We tested the mealybug *Ferrisia gilli* Gullan (Hemiptera: Pseudococcidae) as a vector of GLRaV-1, -2, -3, and -4LV. *Ferrisia gilli* was originally described as a pest on pistachios and almonds in California's San Joaquin Valley (Gullan et al. 2003), and has since become an emerging pest on grape, *Vitis vinifera* L., in California's Sierra Foothill region (Daane et al. 2012). Because this mealybug is only relatively recently described (Gullan et al. 2003), little is known

about its potential damage to crops, although the pest's seasonal phenology has been studied on pistachios and grapes (Haviland et al. 2012, Wunderlich et al. 2013). In the Sierra Foothill region, *Ps. maritimus* and *F. gilli* are the two common mealybug species in vineyards, but it is currently unknown if *F. gilli* is a vector of GLRaVs. *Ferrisia gilli*'s presence in this expanding grape region, and its potential to increase both in population size and geographic range, warrant an investigation of *F. gilli* as a possible vector of virus species that cause GLD.

Materials and Methods

Insects

Third-instar *F. gilli* nymphs were collected from shoots and lower leaves of grapevines in El Dorado County in the Sierra Foothill region in June 2011 and 2012. Insect specimens were confirmed as *F. gilli* by multiplex PCR following Daane et al. (2011), and subsamples of the field-collected insects and their host grapevines tested negative for GLRaV-1, -2, -3, and -4LV by RT-PCR (see below). Collected *F. gilli* were reared on virus-free *V. vinifera* cv. Cabernet Sauvignon cuttings in the Insectary and Quarantine Facility at the University of California, Berkeley, maintained at 24 ± 2°C and a photoperiod of 16:8 (L:D) h. First-instar nymphs emerged from adult females ~2 wk after collection, and were immediately used in the acquisition and transmission experiments described below.

Grapevines

Virus-negative *V. vinifera* cv. Cabernet Sauvignon (clone 5) and several virus-infected accessions (LR101, LR 102, LR106, LR109, LR118, and LV89-01) were grown from cuttings provided by Foundation Plant Services, University of California Davis, CA, and maintained inside fine-mesh cages (BugDorm 44545, Megaview Scientific, Taiwan) during all experiments. All vines were grown in 0.72-liter square plastic pots containing a 4:1 mixture of Supersoil potting medium (Scott's Corporation, Marysville, OH) to perlite potting medium, in a greenhouse maintained at 25 ± 5°C with natural light supplemented by grow-lights for a photoperiod of 16:8 (L:D) h. Vines were regularly fertilized with a 20-20-20 (nitrogen, phosphorus, and potassium) fertilizer at the labeled rate. Presence or absence of GLRaV-1, -2, -3, and -4LV was determined in all experimental source and recipient test plants prior to the start of acquisition and transmission experiments using RT-PCR.

GLRaV Detection

Viral RNA was extracted from plant material as described in Osman et al. (2007) and from insects using TriZol reagent as described in Tsai et al. (2008), then amplified with a Qiagen OneStep RT-PCR kit (Qiagen, Germantown, MD) using multiplexed reactions to test for GLRaV-1, -2, -3, and -4LV (Table 1). Thermal cycler conditions were as follows: reverse transcription at 50°C for 30 min; initial polymerase activation at 95°C for 15 min; 35 cycles of denaturing at 94°C for 0.5 min, annealing at 56°C for 0.5 min, extension at 72°C for 1 min, and final extension at 72°C for 10 min (Tsai et al. 2008). PCR products were visualized on a 2.5% agarose gel stained with ethidium bromide using a GelDoc imager (BioRad, Hercules, CA). Samples that tested positive for GLRaV on at least two of the three collection dates were considered positive. We note that our approach may have resulted in slightly underestimating transmission efficiency. However, avoiding false detection of GLRaV transmission could translate to reduced unnecessary pest treatment applications.

GLRaV Acquisition and Transmission Experiments

To set up virus acquisition, virus-infected grapevines of each accession were thoroughly colonized with first-instar nymphs and isolated in clear plastic cylindrical cages that had fine mesh window and top openings, designed and made for this study. For negative controls, virus-free *V. vinifera* cv. Cabernet Sauvignon (clone FPS 05) plants were infested with *F. gilli* from the virus-free rearing colony and maintained alongside the experimental recipient test plants in the quarantine greenhouse.

After virus acquisition access periods (AAP) of either 2 or 8 d, a leaf piece from a virus source plant with ~50 first-instar nymphs was secured on a leaf of a recipient test plant. First-instar nymphs were confined for a 4-d inoculation access period (IAP). In some experiments, 15 mealybugs were collected from each recipient test plant following the 4-d IAP and tested for GLRaV (results shown in Table 2). More mealybugs survived the 2-d AAP (42 ± 2) compared with the 8-d AAP (31 ± 2) (two sample *t*-test $P = 0.0007$, $df = 1$, 83). To test virus acquisition from infected source plants, 50 *F. gilli* were removed from the virus source plant after an 8-d AAP and tested for GLRaV presence using RT-PCR.

At the end of the IAP for all experimental recipient test plants all remaining mealybugs were killed and plants were washed in soapy water as a contact insecticide. To prevent unwanted GLRaV spread following experimental inoculations, all recipient test plants were inspected 1, 2, 4, and 6 wk after inoculation for any remaining mealybugs. New GLRaV infections can be reliably detected 2 mo after mealybug-mediated inoculations (Tsai et al. 2008). Petiole samples were collected and tested for GLRaV infection 2, 3, and 4 mo after inoculation; on each sample date three or more petioles were collected from each plant and pooled for sample processing. Immediately following collection, all insect and plant samples were stored at −80°C until molecular diagnostic testing.

Mealybug transmission efficiency was estimated for GLRaV-3 using accession LR101 and 14 recipient test plants, inoculated with one group of five insects per recipient plant. The transmission rate was then estimated from the proportion of infected plants as described by Swallow (1985). Five recipient test plants were also inoculated using accession LR101 and one group of five *Pl. ficus* on each test plant. Transmission by *Pl. ficus* has been well documented (Almeida et al. 2013); our inoculations using *Pl. ficus* provided a point of reference for our estimation of transmission efficiency by *F. gilli*. To assess differences in AAP, GLRaV, or mealybug species, chi-square or Fisher exact tests were used to compare the proportion of insect samples or recipient test plants that were positive for GLRaV.

Results

Virus Detection in *F. gilli*

Four GLRaV species were acquired by *F. gilli*. Accession LR101 was infected with GLRaV-3, LR102 with GLRaV-1, -2, and -4LV, LR106 with GLRaV-4LV, LR109 with GLRaV-2 and -3, LR118 with GLRaV-4LV, and finally LV89-01 was infected with GLRaV-2 and -3 (Table 2). GLRaV-1, -2, -3, and -4LV were all detected in *F. gilli* first-instar nymphs after acquisition from these source accessions (Table 2). *Ferrisia gilli* acquired one or more viruses from all source accessions, and overall probability of acquisition did not differ between the 2- and 8-d AAPs ($\chi^2 = 0.20$, $P = 0.72$, $df = 1$). Using groups of 15 insects per sample, 9 of 18 samples were positive for one or more viruses following a 2-d AAP, and 7 of 12 samples tested positive for one or more GLRaV species following an 8-d AAP.

Table 1. Primer pairs used for GLRaV detection

Virus species	Primer names	Reference	Product size (bp)
GLRaV-1	HSP70-149F/293R	(Osman et al. 2007)	168
GLRaV-2	L2F/U2R	(Bertazzon and Angelini 2004)	331
GLRaV-3	LC1F/LC2R	(Osman and Rowhani 2006)	546
GLRaV-4LV	HSPVF/ HSPCR	(Osman et al. 2007)	319
GLRaV4-LV	HSP-26F/HSP-118R	(Osman et al. 2007)	162
GLRaV4-LV	LR9F/ LR9R	(Alkowni et al. 2004)	393

Table 2. Summary of acquisition and transmission experiments performed with *F. gilli*

Source plant accession[a]	GLRaV in source plant	GLRaV in insects	AAP[b]	Acquisition[c]		GLRaV in test plants[d]	Transmission[e]	
				15 insects	50 insects		5 insects	50 insects
LR101	3	3	2	3/4	–	3	1/4	10/16
		3	8	–	10/10	3	4/10	10/13
LR102	1,2,4LV	4LV	2	2/5	–	N	–	0/5
		1,4LV	8	3/5	5/5	N	–	0/5
LR106	4LV	–	2	–	–	4LV	–	1/4
		4LV	8	–	2/5	N	–	0/5
LR109	2,3	2,3	2	2/4	–	3	–	2/4
		2,3	8	2/3	4/4	3	–	3/4
LR118	4LV	–	2	–	–	N	–	0/5
		4LV	8	–	5/5	N	–	0/5
LV89-01	2,3	2,3	2	2/5	–	3	–	4/5
		2,3	8	2/4	4/5	3	–	5/5

[a] Source plant material was from a collection at the University of California, Davis, USA.

[b] Acquisition access period (AAP) was either 2 or 8 d.

[c] Acquisition rate is based on the proportion of insect samples that tested positive for GLRaV, following the AAP, in groups of 15 insects (collected after 4-d IAP on uninfected recipient plants) or 50 insects (collected immediately after AAP on infected source vines). Numbers of samples that tested positive/total number of samples tested are reported.

[d] GLRaV species that was detected in recipient test plants, indicating transmission.

[e] Transmission rate is based on the proportion of recipient plants, inoculated with 5 or 50 insects, that tested positive for GLRaV. Numbers of plants that tested positive/total number of plants tested are reported.

Furthermore, acquisition did not differ among the four GLRaV species ($\chi^2 = 2.13$, $P = 0.55$, df = 3). Following Swallow (1985) and based on the samples in groups of 15 insects, the estimated probability that one insect would acquire at least one GLRaV was 0.05 (5%). When 50 insects per sample were used, 30 of 34 insect samples tested positive for one or more GLRaV; therefore, using this larger inoculation group the estimated probability that one insect would acquire at least one GLRaV was 0.04 (4%); thus both experimental designs led to similar estimates of the probability that an insect will acquire GLRaV.

Virus Detection in Plant Material

No GLRaVs were detected in the negative controls, which included seven groups of *F. gilli* with 15 insects per sample and eight groups with 50 insects per sample. The insects were collected and tested for GLRaV after being allowed to feed on virus-free *V. vinifera* cv. Cabernet Sauvignon plants. Furthermore, none of 17 negative control recipient vines, inoculated with *F. gilli* that fed on virus-free Cabernet Sauvignon plants, became infected with GLRaV.

Of the four GLRaV species acquired by *F. gilli*, only GLRaV-3 and -4LV were transmitted to recipient test plants. No transmission of GLRaV-1 or -2 by *F. gilli* was detected. When each recipient test plant was inoculated with 50 insects following an AAP of 2 d, GLRaV-4LV was transmitted by *F. gilli* to 1 of 29 recipient test plants. GLRaV-3 was transmitted to 34 of 47 recipient test plants. After an AAP of 2 d, 16 of 25 recipient test plants became infected with GLRaV-3, compared with 18 of 22 recipient test plants after an AAP of 8 d, showing the different AAP periods did not significantly affect transmission of GLRaV-3 by *F. gilli* ($\chi^2 = 1.86$, $P = 0.17$, df = 1). GLRaV-3 was the only virus transmitted following an AAP of 8 d (Table 2).

F. gilli and *Pl. ficus* Transmission Efficiencies

Transmission efficiencies of GLRaV-3 by *F. gilli* and *Pl. ficus* were estimated using five insects per recipient test plant, and did not significantly differ from each other (Fisher exact test, $P = 1$). When groups of five insects were used on each recipient test plant, *F. gilli* transmitted GLRaV-3 to 5 of 14 recipient test plants, and *Pl. ficus* transmitted GLRaV-3 to 2 of 5 recipient test plants (Tables 2 and 3). Following Swallow (1985), transmission efficiency of GLRaV-3 by *F. gilli* and *Pl. ficus* were 0.085 and 0.097, respectively. When groups of 50 insects were used on each recipient test plant, GLRaV-3 was transmitted to 8 of 9 recipient test plants by *Pl. ficus* after a 2-d AAP, but to 0 of 5 plants after an 8-d AAP (Fisher exact test, $P = 0.003$; Table 3). Following a 2-d AAP, *Pl. ficus* transmitted GLRaV-3 to 3 of 3 recipient test plants, and transmitted GLRaV-4LV to 5 of 6 recipient plants; thus transmission of the two viruses did not significantly differ from each other (Fisher exact test, $P = 1$; Table 3).

Table 3. Summary of transmission experiments performed with *Pl. ficus*

Source plant accession[a]	GLRaV in source plant	AAP[b]	GLRaV in test plants[c]	Transmission[d]	
				5 insects	50 insects
LR101	3	2	3	2/5	3/3
		8	N	–	0/2
LR106	4LV	2	4LV	–	4/5
		8	N	–	0/3
LR118	4LV	2	4LV	–	1/1
		8	–	–	–

[a] Source plant material was from a collection at the University of California, Davis, USA.

[b] Acquisition access period (AAP) was either 2 or 8 d.

[c] GLRaV species that was detected in recipient test plants, indicating transmission.

[d] Transmission rate is based on the proportion of recipient plants, inoculated with one group of 5 or 50 insects, that tested positive for GLRaV. Numbers of plants that tested positive/total number of plants tested are reported.

Discussion

In our study, virus acquisition efficiency by *F. gilli* did not differ among the GLRaV species. Yet we found that *F. gilli* consistently transmitted GLRaV-3, with little to no transmission of other GLRaV species. Given the previous hypothesis of no virus–vector specificity (Tsai et al. 2010), it was notable that *F. gilli* acquired but did not transmit GLRaV-1, and that transmission efficiency of GLRaV-4LV was low, whereas we found that *Pl. ficus* transmitted both GLRaV-3 and -4LV with no difference between the two virus species. GLRaV-3 is the most prevalent vector-transmissible GLRaV in the Sierra Foothill region, but GLRaV-1 and -4LV are also fairly common; furthermore, mixed GLRaV species infections are prevalent (Sharma et al. 2015). Viral source material in this study was clonal to source material used in studies that found transmission of GLRaV-1, -3, and -4LV by *Pl. ficus*, and GLRaV-3 and -4LV by *Ps. longispinus* (Golino et al. 2002; Tsai et al. 2008, 2010). Therefore, we suspect that larger experiments are needed to consider the transmission of GLRaV-1 and -4LV by *F. gilli*, in order to thoroughly address virus–vector specificity and to inform decisions for comprehensive management of GLRaV species transmission.

Vineyard acreage is rapidly expanding in California's Sierra Foothill region, and *F. gilli is* an important invasive vineyard pest there. *Ferrisia gilli* is especially important given that it is one of just two mealybug species found in the region, the other being *Ps. maritimus* (L. R. Wunderlich, personal observation), which is also an effective vector of GLRaV-3 in vineyards (Blaisdell et al. 2016). *Planococcus ficus* is another invasive pest and well-studied vector of GLRaV species in other California grape-growing regions, which has not yet successfully established in the Sierra Foothill region, perhaps limited by colder winter temperatures in the foothills compared with the interior valley and coastal regions. However, we found similar transmission efficiency of GLRaV-3 by *F. gilli* and *Pl. ficus*, indicating that *F. gilli* may be a worrisomely effective vector of GLRaV-3 in the Sierra Foothill region.

We found reduced GLRaV transmission by *Pl. ficus* after the 8-d AAP compared with the 2-d AAP, but no effect of AAP on transmission by *F. gilli*. It has previously been reported that first-instar nymphs are the most efficient life stage for transmission of GLRaV-3 (Tsai et al. 2008, Le Maguet et al. 2012). *Planococcus ficus* develops more quickly than *F. gilli* (K. M. Daane, unpublished data), and the longer AAP likely resulted in more mature *Pl. ficus* mealybugs being tested, thus leading to the observed reduction in transmission efficiency. During the longer AAP, it is possible that tested insects underwent a molt and nonfeeding period immediately preceding initiation of the IAP. Such a molting nonfeeding period could have led to loss of virus retention in the tested mealybugs due to the semipersistent manner of transmission of GLRaV by mealybugs. For example, *Pl. ficus* transmission of GLRaV-3 reaches a maximum after an AAP of ~24 h with no observed increase resulting from longer AAPs, which is consistent with our findings of no increase in transmission following the extended AAP (Tsai et al. 2008).

Although there is no pheromone to monitor and track *F. gilli* populations, reports from growers indicate that this pest continues to spread throughout El Dorado County of the Sierra Foothill region. Based on current available information, we estimate that the range of *F. gilli* is ~350 acres (L. R. Wunderlich, personal observation). *Ferrisia gilli*'s ability to transmit GLRaV-3 and -4LV elevates the importance and need for improved mealybug monitoring and management in this region, which includes older GLD-infected vineyards that are adjacent to newer installations of "clean" vineyard blocks.

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